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(54) Title: CLONED GLUTAMIC ACID DECARBOXYLASE			
(57) Abstract <p>Isolated polypeptides useful in ameliorating GAD-associated autoimmune disease as well as diagnostic and therapeutic methods of using the peptides are disclosed.</p>			

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CLONED GLUTAMIC ACID DECARBOXYLASE

This application is a continuation-in-part
application of U.S. Serial No. 07/716,909, filed June
5 18, 1991 which is a continuation-in-part application of
U.S. Serial No. 07/586,536, filed September 21, 1990.

BACKGROUND OF THE INVENTION

The present invention was supported by Grant
NS22256 from the National Institutes of Health. The
10 United States Government has certain rights in this
invention.

1. FIELD OF THE INVENTION

The present invention relates to glutamic acid
decarboxylase₆₅ (GAD₆₅) polypeptides and methods of using
15 GAD₆₅ polypeptides diagnostically and therapeutically in
autoimmune disease.

2. DESCRIPTION OF THE BACKGROUND ART

Insulin-dependent diabetes mellitus (IDDM; type I
diabetes) is one of the most common metabolic
20 disorders. In the United States, IDDM affects
approximately one in 300 to 400 people, and
epidemiological studies suggest that its incidence is
increasing. The disease results from the autoimmune
destruction of the insulin-producing β -cells of the
25 pancreas. More specifically, the preonset stage is
characterized by "insulitis", in which lymphocytes
infiltrate the pancreatic islets and selectively
destroy the β -cells. Insulitis may be present for many
years before the onset of clinical symptoms. The
30 typical IDDM presentation of hyperglycemia appears only
after at least 80% of the insulin-producing β -cells are
lost. The remaining β -cells are destroyed during the
next few years.

Although insulin therapy allows most IDDM patients
35 to lead normal lives, this replacement is imperfect and
does not completely restore metabolic homeostasis.
Thus, severe complications which result in dysfunctions

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of the eye, kidney, heart, and other organs are common in IDDM patients undergoing insulin therapy. Because of this, it is highly desirable to extend the latency period and prevent progression (e.g., through
5 administration of immunosuppressant drugs to interfere with the autoimmune process and insulin to achieve better control of the effects of sustained hypoglycemia) between the start of β -cell destruction and the actual requirement of insulin replacement
10 (i.e., when 80% of the β -cells are destroyed). Therefore, a diagnostic test which determines the beginning of β -cell destruction would allow the clinician to administer immunosuppressant drugs
15 (Silverstein, et al., *New England Journal of Medicine*, 319:599-604, 1988) or prophylactic insulin therapy (Keller, et al., *Lancet*, 341:927, 1993) to extend this latency period and thus significantly delay the onset
of insulin replacement side effects.

Many IDDM patients have sera which contain
20 antibodies to a 64kD molecule (Baekkeskov, et al., *J.Clin.Invest.*, 79:926-934, 1987; Atkinson, et al., *Lancet*, 335:1357-1360, 1990), to islet cell cytoplasmic (ICA) molecules or islet cell surface (ICSA) molecules (Bottazzo, et al., *Lancet*, 1:668-672, 1980), or to
25 insulin (Palmer, et al., *Science*, 222:1137-1139, 1983; Atkinson, et al., *Diabetes*, 35:894-898, 1986). Atkinson and coworkers (Atkinson, et al., *Lancet*, 335:1357-1360, 1990) have demonstrated that the presence of antibodies to the 64kD molecule in human
30 sera appears to be the earliest and most reliable indicator that onset of IDDM symptoms will eventually occur.

Recently, Baekkeskov and coworkers established that the 64kD molecule and glutamic acid decarboxylase (GAD)
35 have several antigenic epitopes in common and thus they may be identical or very similar molecules. Although this identification is an important finding, the use of

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this information as a diagnostic tool for predicting IDDM is quite cumbersome and limited unless knowledge of the molecular biology of GAD is known. Studies by Kaufman, et al., (*J. Clin. Invest.*, 89:283, 1992) 5 established that the 64kD molecule was intact GAD₆₅. Consequently, the cloning and subsequent production of large quantities of GAD₆₅ or a GAD molecule which is antigenically substantially identical to the GAD₆₅ molecule or fragments of the GAD₆₅ molecule, both of 10 which can be easily purified, will allow the development of a diagnostic kit designed to predict IDDM as well as effective therapeutic modalities. The present invention provides a means for accomplishing these results.

15 **SUMMARY OF THE INVENTION**

The present invention arose out of the discovery that recombinant DNA technology could be used to produce eukaryotic GAD₆₅ polypeptide and that GAD₆₅ polypeptide could be used in the diagnosis and therapy 20 of patients with autoimmune disease. Particularly relevant is the use of eukaryotic GAD₆₅ polypeptide in the diagnosis and therapy of patients having, or at risk of having, GAD-associated autoimmune disorders such as insulin-dependent diabetes mellitus (IDDM) or 25 stiff man disease.

A major advantage of the present invention is that it provides the art with a ready source of eukaryotic GAD₆₅ polypeptide corresponding to that purified from natural sources, while avoiding the problems associated 30 with the isolation of naturally occurring eukaryotic GAD₆₅ polypeptide when separating it from other eukaryotic non-GAD₆₅ polypeptides. This absence of other eukaryotic non-GAD₆₅ polypeptides is significant in that it allows the development of test systems which 35 will only detect antibodies specifically reactive with GAD₆₅ polypeptides.

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Another advantage of providing eukaryotic GAD₆₅ polypeptide in host cells is that by so doing, it is possible to obtain much larger quantities of the polypeptide than are currently practicably available from natural sources. As a consequence, not only is it possible to use the polypeptide of the invention to more accurately classify and treat patients with such autoimmune diseases as IDDM, but it is also now possible to provide commercially useful quantities of GAD polypeptide for use in diagnostic systems and pharmaceutical compositions.

DESCRIPTION OF THE DRAWINGS

FIGURE 1 Cloning strategy for obtaining GAD₆₅ and GAD₆₇ specific cDNA probes.

FIGURE 2 DNA sequence and corresponding amino acid sequence for rat GAD₆₅.

FIGURE 3 DNA sequence and corresponding amino acid sequence for human GAD₆₅.

FIGURE 4 Comparison of rat GAD₆₅ and human GAD₆₅ amino acid sequences.

FIGURE 5 GAD₆₅ and GAD₆₇ cDNAs hybridize to different size RNAs.

FIGURE 6 Southern blots hybridized with CDNA probes specific for GAD₆₅ and GAD₆₇.

FIGURE 7 Immunological identification of GAD₆₅ and GAD₆₇.

FIGURE 8 Proliferative T-cell responses of NOD mice to β cell antigens.

FIGURE 9 Characterization of GAD specific T-cell response of NOD mice as primed Th1 cells by enhanced clonal size (a) and cell surface markers (b) and IFN γ secretion.

FIGURE 10 Intramolecular spreading of T-cell autoimmunity within the GAD molecule.

FIGURE 11 Delay of onset of IDDM following immunization with GAD₆₅.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the manipulation of genetic materials by recombinant DNA procedures which make possible the production of polypeptides possessing part or all of the primary structural conformation for one or more of the epitopes for binding autoantibodies to glutamic acid decarboxylase₆₅ (GAD₆₅) and for polypeptides that bind to MHC receptors to block T-cell recognition. These polypeptides are highly useful for the immunological detection of autoantibodies reactive with them, since such autoantibodies are pre-diagnostic and indicative of autoimmune diseases such as insulin dependent diabetes mellitus and "stiff man" syndrome. These polypeptides can also be used for purposes of screening drugs, such as those that alter GAD function, and for generation of polyclonal and monoclonal antibodies which, in turn, can be used diagnostically to detect GAD₆₅.

The development of specific DNA sequences encoding eukaryotic GAD₆₅ polypeptide for splicing into DNA vectors can be accomplished using a variety of techniques. For example, alternative methods which can be employed include (1) the isolation of a double stranded DNA sequence from the genomic DNA of the eukaryote; (2) the chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and (3) the *in vitro* synthesis of a double stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA.

The manufacture of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct manufacture of DNA sequences is not possible and the method of choice is the formation of cDNA sequences.

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Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells
5 that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known,
10 the production of labeled single or double stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been
15 denatured into a single stranded form (Jay, et al., *Nucleic Acid Research*, 11:2325, 1983).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes wherein each is potentially the complete complement of a specific DNA sequence in the hybridization sample which includes a heterogeneous mixture of denatured double stranded DNA.
20 For such screening, hybridization is preferably performed on either single stranded DNA or denatured double stranded DNA. These procedures are particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present.
25 In other words, by using stringent hybridization conditions directed toward avoidance of non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement
30 (Wallace, et al., *Nucleic Acid Research*, 9:879, 1981).
35

In addition, a GAD cDNA library can be screened by injecting the various cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the

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desired cDNA expression product, for example, by using antibody specific for GAD₆₅ polypeptide, by using functional assays for GAD₆₅ enzymatic activity, or by measuring the ability of the expression product to stimulate pathogenic T-cells.

Alternatively, a cDNA library can be screened indirectly for GAD₆₅ peptides having at least one epitope using antibodies to GAD₆₅ (Chang and Gottlieb, *J.Neurosci.*, 8:2123, 1988). Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of GAD₆₅ cDNA. Preferred are antibodies directed to an epitope found in the first 100 amino acids of the N-terminal portion of GAD₆₅.

Of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures, the use of genomic DNA isolates, is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides because of the presence of introns.

The present invention provides novel polypeptides of GAD₆₅ which have part or all of the primary structural conformation, that is, a continuous sequence of amino acid residues, having at least one epitope for antibodies to GAD₆₅ or at least one determinant for T-cell recognition. It is possible to use the polypeptide fragments of the invention rather than intact GAD to detect autoantibodies to GAD. The term "polypeptide," as applied to GAD polypeptide, includes any sequence of amino acids having an epitope for autoantibodies to GAD or binds to a T-cell MHC receptor. Thus, the polypeptide fragments of GAD encompassed by the invention possess a biological activity such as the ability to induce and/or bind autoantibodies to GAD, bind to T-cell MHC receptors (especially receptors on pathogenic T-cells) and the like.

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The polypeptides resulting from microbial expression of the DNA sequences of the invention or from other synthetic techniques, such as solid-phase peptide synthesis, can be further characterized by
5 their freedom from association with other eukaryotic polypeptides or other contaminants which might otherwise be associated with GAD in its natural cellular environment or in such extracellular fluids as plasma or urine.

10 Studies by the present inventors unequivocally establish that GAD₆₅ and GAD₆₇ are encoded by distinct genes and are not produced, for example, by post-transcriptional or post-translational modification of a common genomic sequence. Evidence proving that
15 GAD₆₅ and GAD₆₇ are encoded by different genes include:
(a) the largest contiguous sequence of exact identity between GAD₆₅ and GAD₆₇ cDNAs is only 17 nucleotides in length, (b) cDNAs from GAD₆₅ and GAD₆₇ do not cross hybridize with each other's or with each other's mRNA
20 under low stringency conditions (2.0 x SSC, 0.01% SDS, 23°C), and (c) GAD₆₅ and GAD₆₇ cDNAs do not cross hybridize with isolated genomic clones encoding GAD₆₇ and GAD₆₅, respectively.

25 The term "host" includes not only prokaryotes, but also such eukaryotes as yeast, filamentous fungi, plant and animal cells, as well as insect cells which can replicate and express an intron-free DNA sequence of eukaryotic GAD₆₅. However, prokaryotes are preferred as the host organism for screening purposes while
30 eukaryotic cells, especially insect cells, are preferred for expression.

35 The term "prokaryotes" includes all bacteria which can be transformed or transfected with the gene for the expression of GAD₆₅. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, *E. coli*, *S. typhimurium*, *Serratia marcescens* and *Bacillus subtilis*.

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A recombinant DNA molecule coding for the GAD₆₅ polypeptides can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Especially 5 preferred is the use of a plasmid or a virus containing the GAD₆₅ coding sequence for purposes of prokaryotic transformation or transfection, respectively. Alternatively, liposomes containing the DNA of interest can be used to obtain expression in the host (Zhu, et 10 al., *Science*, 261:209, 1993)

Methods for preparing fused, operably linked genes and expressing them in bacteria are well-known in the art (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring 15 Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of GAD₆₅ in prokaryotic hosts.

In general, expression vectors containing promotor sequences which facilitate the efficient transcription 20 of the inserted eukaryotic genetic sequence are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of 25 the transformed cells. The transformed prokaryotic hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptides of the invention can then be isolated from the grown medium, cellular lysates, or 30 cellular membrane fractions.

The isolation and purification of the expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological 35 separations such as those involving the use of monoclonal or polyclonal antibody.

By having provided the sequence of amino acid residues of GAD₆₅, the present invention provides for the manufacture of DNA sequences which code for the

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host expression of polypeptide analogs or derivatives of GAD₆₅ which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues and which share some or all of the 5 epitopes of naturally-occurring polypeptide forms.

The novel DNA sequences of the invention include all sequences useful in providing the expression in prokaryotic or eukaryotic host cells of polypeptides which have at least a part of the primary structural 10 conformation for one or more epitopes capable of reacting with autoantibodies to GAD₆₅ which are comprehended by: (a) the DNA sequence as set forth in Figures 2 or 3 or their complementary strands; (b) DNA sequences which hybridize to DNA sequences defined in 15 (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to DNA sequences defined in (a) and (b) above. Specifically comprehended in (b) are genomic DNA sequences which encode allelic variant forms of 20 GAD₆₅. Part (c) specifically comprehends the manufacture of DNA sequences which encode GAD₆₅, GAD₆₅ fragments, and GAD₆₅ analogs wherein the DNA sequences thereof may incorporate codons which facilitate translation of mRNA in non-vertebrate hosts.

25 Since the cDNA sequence of the invention encodes essentially the entire human or rat GAD₆₅ molecule, it is now a matter of routine to prepare, subclone, and express smaller polypeptide fragments of cDNA from this or a corresponding cDNA sequence which would encode as 30 few as one epitope for autoantibodies to human or rat GAD₆₅. The presence of such an epitope on a cloned polypeptide can then be confirmed using, for example, serum from a patient with autoantibodies to GAD₆₅. An example of such a smaller peptide is the first 35 approximately 100 amino acids from the N-terminus of GAD₆₅ (shown in Figure 3). This amino acid sequence is essentially absent from GAD₆₇. Other examples of specific peptides of the invention are shown in Table 7

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as well as the approximate carboxy-terminal two-thirds of GAD from about amino acid 224 to about amino acid 585. Especially preferred in the carboxy-terminal two-thirds of GAD is the amino acid segment from about 5 amino acid 224 to about amino acid 398.

The present invention further relates to monoclonal antibodies which are specific for the polypeptides of the invention as well as the diagnostic and therapeutic use of these monoclonal antibodies. This specificity 10 enables the monoclonal antibody, and like monoclonal antibodies with like specificity, to be used to bind the polypeptide of the invention when the polypeptide, or amino acids comprising the polypeptide, are present in specimens or a host, such as a human.

Numerous techniques can be utilized to produce the 15 monoclonal antibodies of the invention without resorting to undue experimentation. To a great extent, the products of such monoclonal antibodies is rendered routine because of the highly defined nature of the 20 polypeptides of the invention. Thus, whether the polypeptides of the invention are used for immunization and/or screening, the very limited number of 25 immunogenic determinants on the polypeptides greatly simplifies the identification of cell lines producing monoclonal antibodies of the invention, for example, by limiting the repertoire of clonal expression possible.

One very useful type of cell line for expression of 30 the monoclonal antibodies of the invention is the hybridoma. The general method used for production of hybridomas producing monoclonal antibody is well known (Kohler and Milstein, *Nature*, 256:495, 1975). The resulting hybridomas were then screened for production 35 of monoclonal antibodies capable of binding to the polypeptides of the invention.

The techniques of sensitization and/or 35 immunization, cell fusion, ascites production, selection of mixed hybridomas, or subcloning of monoclonal hybridomas are generally well known in the art. Attention is directed to Koprowski, et al., U.S.

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Patent No. 4,172,124, Koprowski, et al., U.S. Patent
No. 4,196,265, or Douillard, J.Y. and Hoffman, T.,
Basic Facts about Hybridomas, in *Compendium of
Immunology*, Vol. II, L. Schwartz, ed. (1981), which are
5 herein incorporated by reference. In general, the
purified peptides can be modified to have a cystine
attached at the C-terminus to permit unidirectional
attachment of the synthetic peptide to an immunogenic
protein through a connecting bridge, for example,
10 maleimidobenzoylated (MB)-keyhole limpet hemocyanin
(KLH). Other immunogenic conjugates can also be used,
for example, albumin, and the like. The resulting
structure may have several peptide structures linked to
one molecule of protein.
15 Somatic cells derived from a host immunized against
the synthetic peptides can be obtained by any suitable
immunization technique. The host subject is immunized
by administering the antigen, usually in the form of a
protein conjugate, as indicated above, by any suitable
20 method, preferably by injection, either
intraperitoneally, intravenously, subcutaneously, or by
intra-foot pad. Adjuvants may be included in the
immunization protocol.
The initial immunization with the protein bound
25 antigen can be followed by several booster injections
given periodically at intervals of several weeks. The
antibody contained in the plasma of each host can then
be tested for its reactivity with the immunizing
polypeptide of the invention. The host having the
30 highest response is usually most desirable as the donor
of the antibody secreting somatic cells used in the
production of hybridomas. Alternatively,
hyperimmunization can be effected by repeatedly
35 injecting additional amounts of peptide-protein
conjugate by intravenous and/or intraperitoneal route.

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The isolation of hybridomas producing monoclonal antibodies of the invention can be accomplished using routine screening techniques which permit determination of the elementary reaction pattern of the monoclonal antibody of interest. Thus, if a monoclonal antibody being tested binds with a polypeptide of the invention, then the antibody being tested and the antibody produced by the hybridomas of the invention are equivalent.

Alternatively, since the invention teaches polypeptides or amino acid sequences which are specifically required for binding of the preferred monoclonal antibodies of the invention, it is now possible to use these peptides for purposes of immunization to produce hybridomas which, in turn, produce monoclonal antibodies specific for the polypeptide. This approach has the added advantage of decreasing the repertoire of monoclonal antibodies generated by limiting the number of antigenic determinants presented at immunization by the polypeptide. The monoclonal antibodies produced by this method can be screened for specificity using standard techniques, for example, by binding polypeptide to a microtiter plate and measuring binding of the monoclonal antibody by an ELISA assay.

It is also possible to determine, without undue experimentation, if a monoclonal antibody has the same specificity as a monoclonal antibody of the invention by ascertaining whether the former prevents the latter from binding the polypeptide of the invention. If the monoclonal antibody being tested competes with the monoclonal antibody of the invention, as shown by a decrease in binding by the monoclonal antibody of the invention, then it is likely that the two monoclonal antibodies bind to the same, or a closely related, epitope.

Still another way to determine whether a monoclonal antibody has the specificity of a monoclonal antibody of the invention is to pre-incubate the monoclonal

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antibody of the invention with the polypeptide of the invention with which it is normally reactive, and then add the monoclonal antibody being tested to determine if the monoclonal antibody being tested is inhibited in
5 its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of the invention.

10 The GAD₆₅ of the invention is particularly suited for use in immunoassays in which it can be utilized in liquid phase or bound to a solid phase carrier. In addition, GAD₆₅ used in these assays can be detectably labeled in various ways.

15 Examples of immunoassays which can utilize the GAD₆₅ of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA), the sandwich (immunometric assay) and the
20 Western blot assay. Detection of antibodies which bind to the GAD₆₅ of the invention can be done utilizing immunoassays which run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. The concentration of
25 GAD₆₅ which is used will vary depending on the type of immunoassay and nature of the detectable label which is used. However, regardless of the type of immunoassay which is used, the concentration of GAD₆₅ utilized can be readily determined by one of ordinary skill in the
30 art using routine experimentation.

The GAD and GAD fragments of the invention can be bound to many different carriers and used to detect the presence of antibody specifically reactive with the polypeptide. Alternatively, the carrier-bound GAD and
35 GAD fragments can be used therapeutically for extracorporeal absorption of autoimmune antibodies in patients having, or at risk of having, GAD-associated disorders. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene,

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polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding GAD₆₅, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds.

Alternatively, the polypeptide of the invention which comprises the GAD enzymatic domain can be used to detect antibodies to GAD by measuring GAD enzymatic activity. For example, GAD₆₅ and a specimen suspected of having antibodies to GAD₆₅ can be incubated for a period of time and under conditions sufficient to allow binding to occur between GAD₆₅ and the antibodies. The reaction product is precipitated and then tested for GAD enzymatic activity.

For purposes of the invention, the antibody which binds to GAD₆₅ of the invention may be present in various biological fluids and tissues. Any sample containing a detectable amount of antibodies to GAD₆₅ can be used. Normally, a sample is a liquid such as urine, saliva, cerebrospinal fluid, blood, serum and the like, or a solid or semi-solid such as tissue, feces and the like.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes and the like, each of the container means comprising one of the separate elements to be used in the method. For example, one of the container means may comprise GAD₆₅.

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bound to a carrier. A second container may comprise soluble, detectably-labeled second antibody, in lyophilized form or in solution.

In addition, the carrier means may also contain a plurality of containers each of which comprises different, predetermined amounts of GAD₆₅. These latter containers can then be used to prepare a standard curve into which can be interpolated the results obtained from the sample containing the unknown amount of autoantibodies to GAD₆₅.

In using the kit all the user has to do is add, to a container, a premeasured amount of a sample containing a measurable, yet unknown amount of autoantibodies to GAD₆₅ to be detected, a premeasured amount of carrier-bound GAD₆₅ present in the first container, and a premeasured amount of the detectably labeled second antibody present in the second container. Alternatively, the non-detectably labeled GAD₆₅ can be provided attached to the container to which the sample and the detectably labeled second antibody are added. After an appropriate time for incubation, an immune complex is formed and is separated from the supernatant fluid, and the immune complex or the supernatant fluid are detected, as by radioactive counting or addition of an enzyme substrate, and color development.

In an alternative embodiment, a kit comprising the GAD polypeptide of the invention can be used to detect the stage of GAD-associated autoimmune disease in a patient. As further shown herein, Applicants have discovered that certain GAD peptides or fragments are associated with different levels of progression in the autoimmune disease and that the level of disease process can be ascertained by looking at immune cell proliferative response, such as that of the pathogenic T-cell of the patient.

The term "ameliorate" denotes a lessening of the detrimental effect of the autoimmune response in the patient receiving therapy. The term "therapeutically"

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"effective" means that the amount of GAD₆₅ polypeptide used is of sufficient quantity to ameliorate the cause of disease due to the autoimmune response.

The GAD₆₅ polypeptides, including whole GAD₆₅, of the invention can be used therapeutically in patients having, or at risk of having, an autoimmune response associated with GAD₆₅. Such therapy can be accomplished, for example, by the administration of GAD₆₅ polypeptide to induce tolerance to GAD. Such administration can utilize unlabeled as well as labeled GAD₆₅ polypeptide. When unlabeled GAD₆₅ polypeptide is utilized advantageously, it would be in a form wherein, for example, the GAD₆₅ polypeptides are in fragments which are too small to stimulate an immune response, but large enough to bind, or block, the continuance of the autoimmune response. For example, GAD₆₅ could be digested enzymatically into epitope-sized peptides (typically 5-12 amino acids in length) and thereby bind to Fab binding portions present in the body fluids, or on the surface of immune cells, of the patient with autoimmune disease. Alternatively, peptides having at least one determinant for binding to T-cell MHC receptor can be similarly produced or chemically synthesized.

Alternatively, the GAD₆₅ polypeptides of the invention can be administered labeled with a therapeutic agent. These agents can be coupled either directly or indirectly to the GAD₆₅ polypeptides of the invention. One example of indirect coupling is by use of a spacer moiety. These spacer moieties, in turn, can be either insoluble or soluble (Diener, et al., *Science*, 231:148, 1986) and can be selected to enable drug release from the GAD₆₅ polypeptide at the target site. Examples of therapeutic agents which can be coupled to the GAD₆₅ polypeptides of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins.

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The drugs with which can be conjugated to the GAD₆₅ polypeptides of the invention include compounds which are classically referred to as drugs such as mitomycin C, daunorubicin, and vinblastine.

5 In using radioisotopically conjugated GAD₆₅ polypeptides of the invention for immunotherapy, certain isotopes may be more preferable than others depending on such factors as leukocyte distribution as well as stability and emission. Depending on the 10 autoimmune response, some emitters may be preferable to others. In general, α and β particle-emitting radioisotopes are preferred in immunotherapy. Preferred are short range, high energy α emitters such 15 as ²¹²Bi. Examples of radioisotopes which can be bound to the GAD₆₅ polypeptides of the invention for therapeutic purposes are ¹²⁵I, ¹³¹I, ⁹⁰Y, ⁶⁷Cu, ²¹²Bi, ²¹¹At, ²¹²Pb, ⁴⁷Sc, ¹⁰⁹Pd and ¹⁸⁸Re.

20 Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a toxic lectin which has been used immunotherapeutically. This is accomplished by binding the α -peptide chain of ricin, which is responsible for toxicity, to the 25 antibody molecule to enable site specific delivery of the toxic effect.

20 Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a substance 30 produced by *Corynebacterium diphtheriae* which can be used therapeutically. This toxin consists of an α and β subunit which under proper conditions can be separated. The toxic A component can be bound to GAD₆₅ polypeptide and used for site specific delivery to a 35 leukocyte expressing a receptor for GAD₆₅ polypeptide.

Other therapeutic agents which can be coupled to the GAD₆₅ polypeptides of the invention, as well as ex vivo and in vivo therapeutic protocols, are known, or

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can be easily ascertained, by those of ordinary skill in the art.

The present invention also relates to a polypeptide which can be administered therapeutically to
5 ameliorate, or utilized diagnostically to identify, the disease process in patients having, or at risk of having, this disease. The conventional single-letter code used to represent the various amino acids relates as follows:

10

TABLE 1

15

Phe: F Leu: L Ile: I Met: M
Val: V Ser: S Pro: P Thr: T
Ala: A Tyr: Y His: H Gln: Q
Asn: N Lys: K Asp: D Glu: E
Cys: C Trp: W Arg: R Gly: G

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A polypeptide sequence of the invention was identified by comparing the amino acid sequences of human GAD₆₅, human GAD₆₇, and the P2-C protein of the picornavirus, coxsackie virus. The P2-C polynucleotide plays a role in the virus membrane bound replication complex. These analyses established the presence of an extensive sequence similarity between both GAD₆₅ molecules and the coxsackie virus. A core polypeptide of six contiguous amino acid residues of the GAD₆₅ and P2-C polypeptide are identical in amino acid sequence. Indeed, of the 24 amino acids in the polypeptide, 19 are identical or conserved. In addition, there also exists a high charge density and the presence of a proline residue which would render this region highly antigenic (see Table 2).

TABLE 2
COMPARISON OF AMINO ACID SEQUENCES

<u>Protein</u>	<u>Amino Acid Sequence</u>
Human GAD ₆₇	₂₅ S I M A A R Y K Y F P E V K T K G M A A V P K L ₂₆₁
Human GAD ₈	₂₅ A M M I A R F K M F P E V K E K G M A A L P R L ₂₇₃
Coxsackie Virus P2-C	₂₆ F I E W L K V K I L P E V K E K H E F - L S R L ₃₀

The solid line encloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

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In Table 2, the solid line encloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

5 The discovery of this common polypeptide region supports an etiologic role for "molecular mimicry" in the precipitation of diabetes. Thus, where a patient genetically susceptible to IDDM is infected by a coxsackie virus, the immune response to the similar GAD 10 sequence in the patient's β -cells. The immunological response is maintained by the antigenically similar GAD polypeptides resulting in the eventual destruction of the β -cells and the subsequent presentation of IDDM.

15 At present, it is believed that the destruction of pancreatic β -cells in IDDM is mediated by a cellular autoimmune response. As described herein, a polypeptide of the invention can ameliorate the autoimmune response to GAD. Because of the complexity of autoimmune disease, it is possible to envision 20 numerous possible therapeutic modalities which would allow the polypeptides of the invention to be used to ameliorate such diseases. In one embodiment, it appears that the polypeptides of the invention can be utilized to block recognition by a specific T cell 25 receptor (TCR) or an MHC receptor presenting an autoimmune antigen on the surface of an antigen presenting cell (APC). The inhibition of such recognition might occur, for example, by providing the patient with the polypeptide of the invention which, in 30 turn, can displace the autoimmune antigen being presented in the antigen-cleft of the MHC receptor. However, although not wanting to be bound to a particular theory, it is believed that the polypeptides of the invention probably act to induce or restore a 35 tolerogenic state by direct interaction with the appropriate TCR on the surface of a GAD specific pathogenic T-cell. This latter therapeutic approach of

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direct interaction with the TCR is supported by the examples and suggests that suppression of the autoimmune response can be achieved through induction of high-zone tolerance by use of high concentrations of 5 polypeptide, preferably soluble. Another possible mechanism is that the polypeptide of the invention may play a role in anergizing pathogenic T cells by binding to the T cell MHC receptor, thereby preventing the appropriate costimulatory signal.

10 Alternatively, the polypeptides of the invention could be used to stimulate a T-suppressor cell population in order to restore self-recognition and, thereby, ameliorate the autoimmune disease.

15 Stimulation of T-suppressor cell populations could be achieved, for example, by use of a bi-specific antibody having one variable region specific for an epitope present on the autoimmune antigen residing in the cleft of the MHCII receptor and, a second variable region specific for an epitope present on the CD8⁺ receptor.

20 The production of antibody specific for the polypeptide of the invention is a matter of routine to those of skill in the art, as is the preparation of bi-specific antibodies having specificity for 2 or more epitopes.

25 Polypeptide analogs of the present invention may be designed which will compete for recognition of self-antigens at the level of antigen presentation or induce anergy in T cells, due to a lack of a costimulatory signal. Since MHC molecules contain a single peptide binding site, it is possible to design polypeptides 30 which will bind with high affinity to disease-associated MHC molecules, but will not activate disease-causing T-helper cells. Such polypeptides act as antagonists for self-antigen recognition. In the present invention, support for this mechanism is found 35 in the examples, especially Example 7. Precedent for such an approach arises from observation that a mouse lysozyme polypeptide, itself non-immunogenic, can

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compete for MHC binding with an immunogenic polypeptide from hen-egg white lysozyme and thereby reduce T cell activation by that polypeptide (Adorini, et al., *Nature*, 334:623-625, 1988) as well as studies using T-cell receptor peptides to block formation of complex between T-cells, autoantigen and MHC (Howell, et al., *Science*, 246:668, 1989). Similarly, such a therapeutic approach for screening effective polypeptide analogs has been utilized in such autoimmune diseases as experimental autoimmune encephalomyelitis (EAE) (Wraith, et al., *Cell*, 59:248, 1989; Urban, et al., *Cell*, 59:257, 1989).

The single-letter symbols used to represent the amino acid residues in the polypeptides of the present invention are those symbols commonly used in the art. The peptides of the invention include not only the natural amino acid sequences, but also peptides which are analogs, chemical derivatives, or salts thereof. The term "analog" or "conservative variation" refers to any polypeptide having a substantially identical amino acid sequence to a polypeptide provided herein and in which one or more amino acids have been substituted with chemically similar amino acids. For example, one polar amino acid, such as glycine or serine, may be substituted for another polar amino acid; or one acidic amino acid, such as aspartic acid may be substituted for another acidic amino acid, such as glutamic acid; or a basic amino acid, such as lysine, arginine, or histidine may be substituted for another basic amino acid; or a non-polar amino acid such as alanine, leucine, or isoleucine may be substituted for another non-polar amino acid.

The term "analog" or "conservative variation" also means any polypeptide which has one or more amino acids deleted from or added to a polypeptide of the present invention, but which still retains a substantial amino acid sequence homology to such peptide. A substantial

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sequence homology is any homology greater than 70%, preferably at least about 80%, and more preferably at least about 90%. The term "fragment" also means any shorter version of the polypeptides identified herein 5 having at least 6 amino acid residues, wherein the fragment possesses biological activity, or is a fragment capable of inhibiting the stimulation of T-cells by a stimulating polypeptide fragment or substantially full-length molecule.

10 The term "chemical derivative" means any polypeptide derived from a polypeptide of the present invention and in which one or more amino acids have been chemically derivatized by reaction of the functional side groups of amino acid residues present 15 in the polypeptide. Thus, a "chemical derivative" is a polypeptide that is derived from the sequences or polypeptides identified herein by one or more chemical steps. Such derivatized molecules include, for example, those molecules in which free amino groups 20 have been derivatized to form amine hydrochlorides, p-toluene sulfoamides, benzoxycarboamides, T-butyloxycarboamides, thiourethane-type derivatives, trifluoroacetylalamides, chloroaceamides, or formamides. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. 25 Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are 30 those polypeptides which contain one or more naturally occurring amino acids derivatives of the 20 standard amino acids. For example, 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be 35 substituted for histidine; homoserine may be substituted for serine, and ornithine may be substituted for lysine.

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It should be understood that the present invention is not limited to the illustrative polypeptides depicted in Table 2 and Table 9, instead, a polypeptide falling within the scope of this invention may extend 5 outside of, or comprise less than, the region between amino acid 28 and amino acid 50 of coxsackie virus P2-C, or between amino acid 250 and amino acid 273 of GAD₆₅, or between amino acid 258 and amino acid 281 of GAD₆₇, as well as the region between amino acid 78 and 10 amino acid 97, or between amino acid 247 and amino acid 266, or between amino acid 335 and amino acid 356, or between amino acid 479 and amino acid 498, or between amino acid 509 and amino acid 528, or between amino acid 524 and amino acid 543, or between amino acid 539 15 and amino acid 556, or between amino acid 564 and amino acid 583 of GAD₆₅, as long as a substantial part of a given polypeptide is characterized by an amino acid sequence from that region, or segments or combinations thereof, and the polypeptide demonstrates the desired 20 immunological or biological activity against autoimmune disease. In addition, polypeptides according to this invention include those having amino acid sequences which are longer or shorter in length than those of the polypeptides illustrated in Table 2 and Table 9, or 25 which comprise segments or combinations thereof, as long as such polypeptides consist substantially of the region between the amino acids illustrated in Table 2 and Table 9 and demonstrate immunological or biological activity. All polypeptides of the invention should not 30 stimulate or enhance the autoimmune disease.

Accordingly, it should be understood that the specific selection of any one polypeptide within the polypeptides of the invention does not involve undue experimentation. Such a selection may be carried out 35 by taking a number of polypeptides and testing them for their immunological and biological activity in ameliorating the autoimmune disease or for detecting

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antibody. The NOD mouse represents an excellent and well characterized model for screening polypeptides of the invention capable of ameliorating or preventing diabetes. Example 7 illustrates an acceptable 5 procedure for routine screening of candidate polypeptides with biologic activity.

The polypeptides according to the present invention may be prepared by recombinant techniques or by conventional synthesis using known polypeptide 10 synthetic methods, including synthesis on a solid support. An example of a suitable solid phase synthetic technique is that described by Merriweather (J.Am.Chem.Soc., 85:2149, 1963). Other polypeptide synthetic techniques may be found, for example, in 15 Bodanszky, et al., *Peptide Synthesis*, John Wiley & Sons, 2d ed., 1976, as well as other references known to those skilled in the art. A summary of polypeptide synthesis techniques can be found in Stewart, et al., *Solid Phase Peptide Synthesis*, Pierce Chemical Company, 20 Inc., Rockford, Ill., 1984. The synthesis of polypeptides by solution methods may also be used, for example, as described in *The Proteins*, Vol. II, 3d ed., Neurath, et al., eds., 105, Academic Press, New York, NY, 1976. Appropriate protective groups for use in 25 such synthesis can be found in the above references as well as in J. McOmie, *Protective Groups in Organic Chemistry*, Plenum Press, New York, NY, 1973.

The polypeptides of the invention may also be prepared in an appropriate host transformed with DNA 30 sequences that code for the desired polypeptide. For example, a polypeptide may be prepared by the fermentation of appropriate hosts that have been transformed with and which express a DNA sequence encoding the polypeptide. Alternatively, a DNA 35 sequence encoding several of the polypeptides of this invention may be linked together and those sequences may then be used to transform an appropriate host to

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permit the expression of polypeptides involved in the autoimmune disease.

The dosage ranges for the administration of the GAD polypeptides of the invention are those large enough to produce the desired effect in which the symptoms or cellular destruction of the autoimmune response are ameliorated. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like.

Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications.

Dosage can vary from about $0.1\text{mg}/\text{m}^2$ to about $2000\text{mg}/\text{m}^2$, preferably about $0.1\text{mg}/\text{m}^2$ to about $500\text{mg}/\text{m}^2/\text{dose}$, in one or more dose administrations daily, for one or several days.

The GAD polypeptides of the invention can be administered parenterally by injection or by gradual perfusion over time. The GAD polypeptides of the invention can be administered intravenously, intraperitoneally, intra-muscularly, subcutaneously, intracavity, transdermally, intranasally, or enterally.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dex-

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trose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

5 The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the GAD₆₅ polypeptides of the invention, the medicament being used for therapy of autoimmune response to GAD₆₅.

10 The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the
15 scope of the invention.

EXAMPLE 1
CLONING AND EXPRESSION OF GAD₆₅

20 A. RECOMBINANT DNA PROCEDURES

In order to obtain cDNA probes specific for GAD₆₅ and GAD₆₇, total RNA was extracted from adult rat brain by guanidine isothiocyanate-cesium gradient using the method of Chirgwin, et al. (*Biochemistry*, 18:5294, 25 1979). Poly (A) RNA was purified on oligo dT cellulose, using the protocol by Bethesda Research Laboratories (BRL). First strand synthesis was performed by using MMLV-reverse transcriptase (BRL), with conditions suggested, except that poly d(N₆)_n-mers (Pharmacia) were used as primers. This cDNA-RNA mixture was heat inactivated at 65°C for 15 min and stored at -20°C. For PCR, 1/50 of the sample was added to the 100μl reaction. Degenerate oligonucleotides were synthesized (Applied Biosystems) to encode the 30 underlined common amino acid sequences of feline (from cDNA) (Kobayashi, et al., *J.Neurosci.*, 7:2768, 1987) and rat (from peptides) (Chang and Gottlieb,
35

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J.Neurosci., 8:2123, 1988) GAD (Figure 1). The 5'-end sequence of each degenerate oligonucleotide contained one strand of the DNA sequence recognized by either SstI and HindIII (5' oligo) or SstI and SstII (3'-end oligo). These primers were used for selective amplification by polymerase chain reaction of the generated cDNA template as described by Gould, et al. (Proc.Natl.Acad.Sci.,USA, 86:1934, 1989). PCR products were subcloned into HindIII/SstI double digested Bluescript SK vector (Stratagene), transformed into DH5 (BRL), and plated by standard methods (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Colony hybridization was done with an 5'-³²P end labeled oligonucleotide specific to feline GAD₆₇ (Kobayashi, et al., J.Neurosci., 7:2768, 1987). End labeling of oligonucleotide, hybridization conditions, and washing conditions were done as described (Wallace, et al., in *Guide to Molecular Cloning Techniques*; Berger, et al., Eds. in *Methods of Enzymology*; Abelson, et al., Eds. Academic Press, Inc., San Diego, 432-442, 1987), except that the nitrocellulose filters were washed at 50°C for 15 min. Colonies which were positive and negative in the hybridization were individually picked and grown overnight in Terrific Broth (Tartof, et al., Focus, 9:12, 1987). DNA was isolated using a boiling method (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989) and templates were denatured by 0.2N NaOH and purified by Sephadryl S400 spun columns (Pharmacia). Sequencing of denatured double stranded template was by the chain-termination method (Sanger, et al., Proc.Natl.Acad.Sci.,USA, 74:5463, 1977) using the T7-sequencing kit (Pharmacia).

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As shown in Figure 1, PCR-generated rat GAD₆₅ and GAD₆₇ cDNAs were used as probes to screen a lambda ZAP (Stratagene) rat hippocampus library provided by S. Heinemann (Salk Institute) by standard techniques
5 (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). A 2400 nucleotide GAD₆₅ cDNA (the largest clone) was isolated and subcloned by "zapping" as described by Stratagene. When a rat GAD₆₇ cDNA was obtained which was smaller than a 3.2kb rat GAD₆₇ cDNA clone already on hand, the larger cDNA was sequenced.
10 Exo III deletions (Henikoff, *Gene*, 28:351, 1984) were made in both directions for GAD₆₅ and GAD₆₇ and templates were prepared and sequenced as described above.
15 Anchored PCR (Frohman, et al., *Proc.Natl.Acad.Sci.,USA*, 85:8998, 1988) was done to clone the remaining 5'-ends of GAD₆₅ and GAD₆₇ mRNAs which were not represented in the original cDNA clones isolated in the library screening. Sequencing of these clones revealed that
20 neither GAD₆₅ nor GAD₆₇ mRNAs contained any further initiation codons (AUGs) in frame with the previously designated initiation codons of the original cDNA clones.

25

EXAMPLE 2

CHARACTERIZATION OF CLONED GAD₆₅

A. NORTHERN BLOT HYBRIDIZATION

Two PCR-derived cDNA probes were hybridized to
30 Northern blots containing rat brain RNA in order to determine whether the GAD₆₇ and GAD₆₅ cDNAs were derived from two different mRNAs. RNA was extracted as described in Example 1. Poly (A) RNA was separated by electrophoresis in formaldehyde and transferred onto
35 Biotrans (ICN) membranes, and hybridization was performed as described by Well, et al. (*J.Neurosci.*, 16:311, 1986), except that 100μl/ml of poly (A) was

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added. Probes were labeled to approximately 10^9 dpm/ μ g by the oligolabeling procedure of Feinberg and Vogelstein (*Anal.Biochem.*, 132:6, 1983). Identical results were subsequently obtained with full-length clones of GAD₆₅ and GAD₆₇ cDNAs.

5 As shown in Figure 5, lanes 1 and 2 contain 1 μ g of poly (A) selected RNA extracted from rat cerebellum. Lane 1 was hybridized to a cDNA probe for the rat cognate of feline GAD₆₇ (Kobayashi, et al., *J.Neurosci.*, 7:2768, 1987) and lane 2 with a cDNA probe for the rat peptide sequence (which corresponds to GAD₆₅).

10 The cDNA probe for the rat peptide sequence hybridized to a 5.7kb RNA, while the cDNA probe for the rat cognate of feline GAD₆₇ cDNA, hybridized to a 3.7kb RNA. This demonstrates that GAD₆₅ and GAD₆₇ are not 15 derived from the same mRNA.

B. GENOMIC HYBRIDIZATION OF GAD₆₇ AND GAD₆₅

20 In order to investigate the possibility that GAD₆₇ and GAD₆₅ arise from separate genes, cDNAs of both GAD₆₇ and GAD₆₅ were hybridized to DNA blots containing genomic DNA.

25 For Southern blots, genomic DNA was extracted from rat liver as described (Kaiser, et al., in *DNA Cloning*, vol.I, A Practical Approach, D.M. Glover ed., IRL Press, Oxford, pp. 38-40, 1985). DNA (10 μ g/sample) was digested to completion with EcoRI and HindIII using conditions recommended by the suppliers (BRL, Gaithersburg, MD). DNA fragments were separated by 30 electrophoresis at 1.5v/cm for 16 hrs in 0.8% agarose. The DNA was then transferred to Zeta-Probe membranes (Bio-Rad), hybridized, and washed, as described by Gatti, et al. (*Biotechniques*, 2:148, 1984), except that 5 μ g/ml Carnation dried milk was substituted for 35 Denhardt's solution. Probes for Southern blots were labeled as described in Example 1, above.

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As shown in Figure 6, genomic DNA digested with HindIII and EcoRI are in lanes 1 and 3 and lanes 2 and 4, respectively. GAD₆₇ cDNA was hybridized to lanes 1 and 2, whereas GAD₆₅ cDNA was hybridized to lanes 3 and 4. Numbers along the side of the gel are the DNA fragment sizes in kilobases.

This data shows that the two cDNAs hybridize to genomic fragments of different sizes. In addition, the greatest contiguous stretch of identical nucleotide sequence of GAD₆₅ and GAD₆₇ cDNAs is only 17 nucleotide bases in length. Thus, GAD₆₇ and GAD₆₅ are encoded by two distinct genes.

C. ENZYMATIC COMPARISON OF GAD₆₇ AND GAD₆₅

Studies were done comparing the effect of PLP on the activity of GAD₆₇ and GAD₆₅. In so doing, both cDNAs were subcloned into vectors that allowed their expression in bacteria (Studier, et al., *J.Mol.Biol.*, 189:113, 1986). Overexpression of "fusionless" GAD₆₅ and GAD₆₇ was accomplished by subcloning GAD₆₅ cDNA into the NcoI site of pET-8c and GAD₆₇ cDNA into the NheI site of pET-5c vectors (Studier, et al., *J.Mol.Biol.*, 189:113, 1986).

To obtain compatible sticky ends for correct in-frame subcloning of both cDNAs, selective amplification was performed by PCR using conditions suggested by United States Biochemical (USB), with 200μM dNTPs and 1.5mM MgCl₂ in the mixture and annealing at 55°C with 20 cycles to decrease infidelity of AmpliTaq (USB). Primers specific for GAD₆₅ and GAD₆₇ contained one DNA strand of the NcoI and SpeI recognition sites, respectively. Since there is a NheI restriction site within the coding region of GAD₆₇, SpeI (which is compatible with NheI) was used.

PCR products were subcloned into their respective pET vectors, transformed into DH5 and plated as described (Maniatis, et al., *Molecular Cloning: A*

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Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). Colonies were picked and grown overnight in LB broth with 50 μ g/ml ampicillin. Subclones with correct orientation were transformed
5 into BL21(DE3) strain (Studier, et al., *J.Mol.Biol.*, 189:113, 1986) for overexpression. As a negative control, the pET-8C vector with no insert was transformed and subsequently induced. Single colonies were picked, grown, induced by 1mM isopropyl-B-D-
10 thiogalacto-pyranoside (IPTG), and analyzed on SDS-PAGE gels as described (Sambrook, et al., *Molecular Cloning a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 17.15-17.16, 1989).

To measure GAD activity, we induced 10ml cultures
15 of bacteria at OD₆₀₀-0.5 with 1mM IPTG. Two hours after induction, bacteria was spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-aminoethylisothiouronium bromide (AET), and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, *Anal.Biochem.*, 72:248, 1986) in the supernatant (supernatant was stored in aliquots at -70°C). Brain homogenates were
20 prepared as described (Legay, et al., *J.Neurochem.*, 46:1478, 1986). GAD activity was measured as described (Krieger, et al., *J.Neurochem.*, 33:299, 1984) with 0.2mM PLP present or absent and 20 μ l of brain homogenate or bacterial lysate in the incubation
25 mixture. Production of ¹⁴CO₂ in bacterial lysates was linear with respect to time of incubation and protein concentration.
30

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TABLE 3

5	Source	GAD Specific Activity*		Fold Increase in Induction
		- PLP	+ PLP	
	BL21(DE3) + pET-8c	12 ± 0.4	9 ± 1	--
10	BL21(DE3) + pET-GAD ₆₅	115 ± 3	773 ± 61	6.7
	BL21(DE3) + pET-GAD ₆₇	160 ± 2	389 ± 8	2.4
	Rat Brain	131 ± 5	216 ± 2	1.6
15	• cpm's of ¹⁴ CO ₂ /μg protein/hr of triplicates ± S.E.M.			

As shown in Table 3, bacterial lysates containing GAD₆₅ or GAD₆₇ catalyze the conversion of [1-¹⁴C]-glutamate to GABA and ¹⁴CO₂.

PLP stimulates the enzymatic activity of GAD₆₅ more than GAD₆₇. This greater stimulation probably reflects the faster cycling of GAD₆₅ through the inactivation cycle proposed by Martin and coworkers (Martin, 25 *Cell.Mol.Neurobiol.*, 7:237, 1987). This faster cycling suggests that GAD₆₅ contributes more to the pool of apo-GAD that exists *in vivo* (Miller, et al., *Brain Res.Bull.*, 5(Suppl.2):89, 1980). Thus, *in vivo*, PLP appears to regulate GAD₆₅ activity more than GAD₆₇ activity.

GAD₆₅ activity in bacterial lysates is similar to the five-fold PLP stimulation of GAD activity found in synaptosomes prepared from rat substantia nigra (Miller, et al., *J.Neurochem.*, 33:533, 1979). Because both GADs are more dependent upon added PLP in bacteria than is the GAD activity in crude rat brain homogenates, the endogenous PLP concentration of bacteria lysates may be less than rat brain homogenates.

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D. IMMUNOLOGICAL IDENTIFICATION OF GAD₆₅ AND GAD₆₇

Rat brain homogenates and bacterial lysates were extracted as described above. Equal volumes of loading buffer were added to each sample as described (Harlow, et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). Proteins were separated by electrophoresis in a 10% acrylamide gel in SDS and electrophoretically transferred to nitrocellulose (Harlow, et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). The unreacted sites were blocked with a phosphate buffered saline (PBS) solution containing 2% bovine serum albumin (fraction V), 1% gelatin, and 1% Triton-X-100 at 42°C for one hr. After washing, the nitrocellulose filter was then cut into three sections and incubated with the following primary antibodies: lanes 1 to 4 with a 1/2000 dilution of the antiserum of Oertel, et al. (*Neuroscience*, 6:2689, 1981), which recognizes both GAD₆₇ and GAD₆₅; lanes 5-8 with a 1/2000 dilution of K-2 antiserum, which recognizes only GAD₆₇; lanes 9-12 with a 1/2000 dilution of GAD-6 monoclonal antibody, which is specific for GAD₆₅ (Chang, et al., *J.Neurosci.*, 8:2123, 1988). All filters were extensively washed and appropriate secondary antibodies were incubated and washed. Bound antibodies were detected with ¹²⁵I-labeled protein A and autoradiography. Each lane contained the following: lanes 1, 5, and 9 are BL21(DE3) + pET-GAD₆₇; lanes 2, 6, and 10 are BL21(DE3) + pET-GAD₆₅; lanes 3, 7, and 11 are rat brain homogenate; and lanes 4, 8, and 12 are BL21(DE3) + pET-8c.

The immunoblots of bacterially produced GAD₆₅ and GAD₆₇ demonstrated that GAD₆₅ indeed corresponds to the smaller GAD in brain extracts, and GAD₆₇ to the larger form (Figure 7). Previous work has demonstrated the

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correspondence of GAD₆₇ to the larger GAD for feline
GAD₆₇, and for mouse GAD₆₇ (Katarova, et al.,
Eur.J.Neurosci., 2:190, 1990; 235, 1987). The
mobililities of bacterially produced GAD₆₅ and GAD₆₇ (as
5 detected with the antiserum of Oertel, et al.
(*Neuroscience*, 6:2689, 1981) are identical to the
immunoreactive doublet seen in rat brain homogenate.

The smaller molecular weight and larger molecular
10 weight forms of GAD in rat brain are thus identical in
antigenicity and size to the products of GAD₆₅ and GAD₆₇
cDNAs, respectively. Consequently, the two GADs in rat
brain are GAD₆₅ and GAD₆₇. From these data it can also
be concluded that the molecular identity of the
reported PLP-dependent and PLP-independent GADs by
15 Tapia (Bayon, et al., *J.Neurochem.*, 29:519, 1977) are
GAD₆₅ and GAD₆₇, respectively. Martin and coworkers
(Spink, et al., *Brain Res.*, 421:235, 1987) have
reported the existence of four kinetically different
20 forms of rat brain GAD. However, immunoblotting
experiments (with the antisera used here) of these
forms have not been reported.

**E. DISTRIBUTION OF GAD₆₅ AND GAD₆₇ IN RNAs IN BRAIN
TISSUE**

25 Experiments were done to determine the distribution
of GAD₆₅ and GAD₆₇ in RNAs in cerebellum using *in situ*
hybridization.

Transcripts of, respectively, 3.2kb and 2.3kb from
30 GAD₆₅ and GAD₆₇ cDNAs, were radiolabeled with ³⁵S
according to Wuenschell, et al.
(*Proc.Natl.Acad.Sci., USA*, 83:6193, 1986) procedure.
Hydrolyzed fragments of 200 bp were hybridized to
coronal sections of a rat cerebellum. Animals were
anesthetized under halothane and decapitated. The
35 brain was rapidly frozen in dry ice and coronal frozen
sections (12μm) were fixed for 30 min in freshly
prepared 4% formaldehyde in phosphate-buffered saline

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(PBS; 130mM NaCl, 10mM Na phosphate, pH 7.0). The tissue was dehydrated through graded ethanol solutions and stored at -70°C.

In order to increase tissue permeability, the
5 sections were submitted to the following pretreatments:
rehydration through graded ethanol solutions (5 min
each in 95%, 85%, 70%, 50%, and 30% ethanol); PBS (5
min); 0.02N HCl (10 min); PBS (5 min); 0.01% Triton N-
101 in PBS (1 min); PBS (2 x 5 min); 1 μ g/ml proteinase
10 K (7.5 min); and glycine (to inhibit proteinase K) in
PBS (3 x 5 min). Proteinase K was digested for 30 min
at 37°C before use. Sections were then incubated at
37°C in 50% formamide, 750mM NaCl, 25mM EDTA, 0.2% SDS,
0.02% BSA, 0.002% Ficoll, 0.02% polyvinylpyrrolidone,
15 250 μ g/ml yeast tRNA, 250 μ g/ml poly A, and 25mM PPES (pH
6.8).

For the hybridization, 100mM DTT, 10% dextran
sulfate, and sense or antisense 35 S-RNA were added to
the prehybridization solution. An aliquot (50 μ l) of
20 the hybridization solution containing about 3 ng (10^6
cpm) of probe (sense or antisense) was added onto the
slides. Each slide was coverslipped and incubated for
16 hrs at 50°C, following which the siliconized
coverslips were removed by brief washing in 4 x SSC (1
25 x SSC - 150mM NaCl, 60mM Na citrate, pH 7.0).

Sections were then treated with ribonuclease A
(50 μ g/ml in 0.5M NaCl, 10mM Na thiosulfate, 1mM EDTA,
10mM TrisHCL, pH 8.0) for 20 min at 37°C and rinsed for
2 hrs at room temperature in 2 x SSC, 10mM Na
30 thiosulfate, for 30 min at 55°C. Sections were
dehydrated in ethanol, delipidated in xylene, coated
with Kodak NTB2 emulsion and exposed for 10 days at
4°C. The emulsion was developed with Kodak D19, and
the tissue counterstained with cresyl violet.

35 Autoradiographic grains were detected using
reflected polarized light and grain numbers, densities,
nd cell areas were determined with an Analytic Imaging

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Concepts image analyzer system. Due to the low background level, the criteria for defining a cell "labeled" was based on the presence of more than 5 clustered grains. The GAD labeled cells were found 5 scattered throughout the brain, enabling the measurement of the number of grains over individual cells. The boundary of the cell and the area covered by a grain allowed the calculation of the number of grains per cell. This analysis was done at a high 10 magnification (800X), using both reflected polarized light and transmitted light to simultaneously visualize the stained cell and the superimposed grains. Numbers 15 are means \pm S.E.M. of "n" cells.

TABLE 4

15

<u>CELL TYPE</u>	<u>GRAINS/CELL</u>		<u>GAD₆₇:GAD₆₅</u>
	<u>GAD₆₇mRNA</u>	<u>GAD₆₅mRNA</u>	
Purkinje	172 \pm 34 (87)*	43 \pm 2 (70)	4.0
Golgi II	96 \pm 8 (80)	64 \pm 9 (65)	1.5
Basket	61 \pm 12 (102)	16 \pm 1 (57)	3.8
Stellate	55 \pm 15 (65)	18 \pm 3 (37)	3.1

* \pm S.E.M. (n)

30 In all neuronal types GAD₆₇ mRNA levels are greater. The observations with *in-situ* hybridization are consistent with previous findings (Nitsch, *J.Neurochem.*, 34:822, 1980; Denner, et al., *J.Neurochem.*, 44:957, 1985; Itoh, et al., *Neurochem. Res.* 6:1283, 1981) that the ratio of PLP dependent to independent GAD activities in the cerebellum is one of the lowest in brain regions tested. In addition, as shown in Table 3, the order of amounts for GAD₆₇ mRNA is Purkinje > Golgi II > Basket > Stellate cells; in 35 contrast, for GAD₆₅ mRNA, this order is Golgi II > Purkinje > Basket > Stellate cells.

40 The expression of GAD₆₅ and GAD₆₇ mRNAs thus differs among classes of neurons. The contribution of each to

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total GAD activity in turn affects how GABA production is regulated. For example, the substantia nigra contains one of the highest ratios of PLP-dependent to PLP-independent GAD activities (Nitsch, *J. Neurochem.*, 5 34:822, 1980). Increasing GABA concentration in the substantia nigra by local injection of inhibitors of GABA catabolism is especially effective in reducing seizure susceptibility (Gale, *Fed. Proc.*, 44:2414, 1985). Experimental animals undergoing seizures induced by PLP-antagonists may therefore be unable to inhibit seizure propagation because of inhibition of GAD₆₅ particularly in nerve terminals within the substantia nigra.

15 F. SUBCELLULAR LOCATION OF GAD₆₅ AND GAD₆₇

The distribution of GAD₆₅ and GAD₆₇ was evaluated in the S₂ and synaptosome subcellular fractions. S₂ is a high speed supernatant consisting of the cytosol of all cells in the brain, while the synaptosomal fraction consists primarily of nerve endings (Gray, et al., *J. Anat.*, Lond, 96:79, 1962). For these studies, whole rat brain fractionation was performed as described by Booth and Clark (Booth, et al., *Biochem. J.*, 176:365, 20 1978). Protein concentrations were determined by Schaffner and Weissman (Schaffner, et al., *Anal. Biochem.* 56:502, 1973). Samples were prepared as described (Kaiser, et al., *DNA Cloning, Vol. I, A Practical Approach*, D.M. Glover ed. (IRL Press, Oxford, 1985, pp. 38-40), and immunoblotting was done as 25 described above using GAD-6 monoclonal antibody and K-2 antiserum. Equal amounts of protein (16 μ g) were added to each lane. Autoradiography showed a linear response of increasing amount of ¹²⁵I-protein A bound to antibody with protein concentrations of 1, 3, 10, 30, 100 μ gs 30 with both K-2 antiserum and GAD-6 monoclonal antibody 35 (data not shown).

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The results showed that GAD₆₇ was present in equal amounts in both fractions. Since the S₂ fraction contains the cytosolic proteins of glial (as well as other non-neuronal) and neuronal cells, the 5 concentration of GAD₆₇ must be greater in neuronal cell bodies than in nerve endings. In contrast, the concentration of GAD₆₅ was greater in synaptosomes than in S₂. These subcellular fractionation experiments suggest that, in contrast to GAD₆₅, a much greater 10 fraction of GAD₆₇ is present in cell bodies of neurons than in nerve terminals. Thus, subcellular fractionation, like immunohistochemistry, shows that GAD₆₅ and GAD₆₇ have different subcellular distributions.

In vivo experiments utilizing inhibitors of GABA 15 synthesis and degradation have suggested that the GABA pool in neuronal cell bodies is different from that in the nerve terminals (Iadarola, et al., *Mol. Cell. Biochem.*, 39:305, 1981). GABA produced by GAD₆₇ may be involved more in cellular metabolism (for example, in 20 the GABA shunt) and in dendrodendritic synapses. The dendrites of granule cells in the olfactory bulb, which form dendrodendritic synapses with mitral dendrites (Shepard, *Physiol. Rev.*, 52:864, 1972) and probably release GABA (McLennan, *Brain Res.*, 29:177-184, 1971), 25 label intensely with K-2 antiserum. While not shown here, it has also been found greater GAD₆₇ than GAD₆₅ mRNA levels (2-3 fold) in the olfactory bulb. This distribution is consistent with the reported finding 30 that most GAD activity in the olfactory bulb is present in S₂ and P₁ (crude nuclear pellet) and not in synaptosomes (Quinn, et al., *J. Neurochem.*, 35:583, 1980).

The differing subcellular distributions of GAD₆₅ and 35 GAD₆₇ could result from cytoskeletal anchoring or from some unknown protein targeting mechanism. Some cytoskeletal proteins have distributions that resemble GAD₆₅ and GAD₆₇. For example, in cultured sympathetic

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neurons Peng, et al. (*J Cell. Biol.*, 102:252, 1986), demonstrate that 84% of tau is in axons while 100% of MAP-2 is in cell bodies and dendrites. In addition, 43kd protein, a cytoskeletal protein, is thought to anchor the acetylcholine receptor to the underlying membrane cytoskeleton (Flucher, et al., *Neuron*, 3:163, 1989).

EXAMPLE 3

10 DETECTION OF GAD AUTOANTIBODIES IN CLINICAL SPECIMENS

A. MATERIALS AND METHODS

15 1. Patient Specimens. Sera from four groups of individuals were selected from a previous study by Atkinson and co-workers (Atkinson, et al., *Lancet*, 335:1357-1360, 1990). These groups consisted of: Group (1), 1 new onset IDD patients diagnosed according to the established National Diabetes Data Group (NDDG) criteria (Gleichman, et al., *Diabetes*, 36:578-584, 1987) that had been referred to the University of Florida, Diabetes Clinics; Group (2), 5 randomly selected islet cell cytoplasmic antibody (ICA) negative non-diabetic controls without any known family history of autoimmune disease; Group (3), 13 individuals whose sera had been collected 3 to 66 months prior to their documented clinical onsets of IDD; Group (4), non-diabetic controls and relatives, and those who were studied prior to their onsets of IDD; and Group (5), 3 patients at risk for IDDM, but where onset has not yet occurred. This latter group had been ascertained through ongoing prospective ICA screening studies of more than 5000 first degree relative of IDD probands, and 8200 individuals from the general population (of which 4813 were school children).

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2. Islet Cell Autoantibodies. ICA were assayed by indirect immunofluorescence on blood group O cryocut pancreatic (Atkinson, et al., *Lancet*, 335:1357-1360, 1990). All results were interpreted on coded samples, 5 with control negative and positive sera in each batch. The degrees of ICA positivity were analyzed with the guidelines established by the Immunology Diabetes Workshop (IDW) for the standardization of ICA (Gleichman, et al., *Diabetes*, 36:578-584, 1987). All 10 positive sera were titered by end point dilution, and the Juvenile Diabetes Foundation (JDF) units were determined by reference to a standard serum previously calibrated to the international JDF standard of 80 units. In the studies reported here, a positive ICA 15 result was defined by replicate titers of 10 JDF units or greater.

3. HLA DR Typing. HLA DR typing was performed as adapted from the method described by Van Rood and Van Leuwen (*Nature*, 262:795-797, 1976), using DR trays (One 20 Lambda Laboratories, Los Angeles, CA).

4. Human Islet Cells. Human pancreatic islets were isolated from cadaveric pancreases and maintained *in vitro* as previously described (Ricordi, et al., *Diabetes*, 37:413-420, 1988). The islet cells were 25 metabolically labeled with ^{35}S methionine (Amersham, Arlington Heights, IL) *in vitro* (95% air/5%CO₂).

5. Islet Cell Extractions and Immunoprecipitations. Islet cells were extracted as previously described by 30 Atkinson, et al. (*Lancet*, 335:1357-1360, 1990) with the following modifications. For immunoprecipitation studies, the islet cell lysates were precleared twice by incubation (2h, 4°C) with either control, IDD serum (100μl), or GAD-6 (Chang, et al., *J.Neuro*, 8:2123-2130, 35 1988) (1μl in 99μl of Tris buffer (Atkinson, et al., *Lancet*, 335:1357-1360, 1990) for every 1000 islets. Immune complexes were then absorbed (1h 4°C) with an

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excess of protein A Sepharose CL-4B (Pharmacia, NJ). Aliquot volumes representing 1000 islet cells containing unbound (precleared) lysate were then incubated (12h, 4°C) with either IDD or control sera (25 μ l), or GAD-6 (Chang, et al., *J.Neuro.*, 8:2123-2130, 1988) (1 μ l in 25 μ l Tris buffer). Following another incubation with protein A Sepharose CL-4B (1h, 4°C), the complexes were then washed 5 times with 50mM Tris HCl (pH 7.4) with 0.1% SDS, 1.0% Triton X-114, and 2mM EDTA, and then washed again one time in double distilled water. The protein A Sepharose CL-4B was then boiled in Laemmli sample buffer (Laemmli, *Nature*, 227:680-685, 1970), and the samples were subjected to SDS-PAGE and fluororadiography (Kodak, X-omat AR5) using Enhance (New England Nuclear). Alternatively, the autoradiographs were analyzed by a BETAGEN (Boston, MA) analyzer. Both 64KA positive and negative sera were used in each assay, to serve as interassay controls. All fluororadiographs were analyzed and rated as positive or negative after comparison with the known interassay controls. Positive serum samples were designated as 1 when a sample resulted in immunoprecipitation of a low intensity 64,000 M_r band, 2 if a moderate intensity band was observed and 3 if the intensity of the immunoprecipitated protein was high. A similar rating procedure was employed for the intensity of bands corresponding to immunoprecipitated ³⁵S-GAD₆₅ and ³⁵S-GAD₆₇.

6. Immunoprecipitations. Immunoprecipitation of bacterial lysates containing ³⁵S-GAD₆₅ or ³⁵S-GAD₆₇, and GAD from human brain homogenate, was completed as described above in immunoprecipitation studies of human islet cell extractions.

7. GAD Assays. Human brain homogenates were incubated with patient sera as described above in human islet cells. After absorption and washes, the protein A agarose slurry was aliquoted into three equal volumes

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and GAD activity was measured as described (Krieger, et al., *Neurochem.* 33:299, 1984). Briefly, Protein A agarose beads were incubated with (1-¹⁴C)-glutamate (Amersham) in a designated incubation mixture (Krieger, et al., *J. Neurochem.* 33:299, 1984) and production of ¹⁴CO₂ was quantitated by a liquid scintillation counter.

5 **8. Production of ³⁵S-GAD₆₅ and ³⁵S-GAD₆₇.** Rat GAD₆₅ and GAD₆₇ cDNAs were subcloned into a bacterial expression system as previously described. Labeling of ³⁵S-GADs

10 was completed by pulsing IPTG induced bacterium (growing in Minimal Media) for 15 minutes with TRAN ³⁵S-label (ICN). Cultures were then spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-

15 aminoethylisothiouronium Bromide (AET) and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, *Anal.Biochem.*, 72:248, 1986) in the supernatant (supernatant was

20 stored in aliquots at -70°C).

B. IMMUNOREACTIVITY OF IDDM SPECIMENS

25 Sera from patients with IDDM were tested for the ability to precipitate GAD from human brain homogenates.

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TABLE 5
DATA FROM IDDM PATIENTS IMMUNOPRECIPITATE AND ACTIVITY

Patient	IDDM	Pre-IDDM Period ¹	64K ²	JDF ³	GAD Activity ⁴ cpm's ⁵
DA	*	>24	3	164	13,762
DC	*	>1	3	20	1,719
RS	+	5	3	40	588
NL	+	0	2	80	440
DM	+	>1	2	10	184
C	na	0	0	0	280
C	na	0	0	0	285
C	na	0	0	0	325
C	na	0	0	0	275
C	na	0	0	0	270

¹ Expressed as months² 64K titers as described in Experimental Methods³ The islet cell antibody test as expressed in Juvenile Diabetes Foundation (JDF) units⁴ Not adjusted for background⁵ At risk for diabetes (also, failed glucose test)

na . Not applicable

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As shown in Table 5, the sera of four (out of five) at risk for IDDM or IDDM patients bound significantly greater amounts of enzymatically active GAD of human brain extracts than sera from control patients. In 5 addition, sera from one of the patients was drawn in a pre-IDDM period, thus autoantibodies to GAD are present prior to the onset of IDDM symptoms (see C below).

Further experiments (results not presented) showed that the sera of two at risk IDDM patients (DA, DC) 10 immunoprecipitated recombinantly produced ^{35}S -GAD₆₅ whereas recombinantly produced ^{35}S -GAD₆₇ was only recognized by sera of patient DA (and to a lesser degree than ^{35}S -GAD₆₅).

Additional studies using patient DA sera showed the 15 presence of antibodies which recognize specific polypeptides produced in human pancreatic islet cells. Electrophoretic analysis of the bound polypeptides demonstrated the presence of autoantibodies to a 64kD component, as previously shown by others in human IDDM 20 (Baekkeskov, et al., *Nature*, 298:167-169, 1982) and in animal models (Baekkeskov, et al., *Science*, 224:1348-1350, 1984; Atkinson, et al., *Diabetes*, 37:1587-1590, 1988). Prior absorption of these sera with GAD-6 monoclonal, which recognized GAD₆₅ but not GAD₆₇, or with 25 bacterially produced GAD₆₅, abolished the ability of the sera to recognize the 64kD pancreatic polypeptide. The epitopes recognized by autoantibodies to the 64kD autoantigen are thus present in GAD₆₅, indicating that the 64kD autoantigen is indeed GAD₆₅. In order to 30 investigate the predictive value of GAD₆₅, sera drawn from patients prior to onset of clinical manifestation of IDDM were tested for autoantibodies to GAD₆₅.

TABLE 6

IDDM PATIENTS ANALYZED FOR AUTOANTIBODIES PRIOR TO THE ONSET OF DISEASE

Patient	Sex	HLA	Age Onset ¹	Pre-IDDM Period ²	IDM ³	GAD ⁴	GAD ⁵
TA	M	3,2	17	11	20	2	0
CA	F	4,5	38	4	0	1	1
RA	M	2,1	5	34	0	2	1
TB	M	2,4	11	66	40	1	1
AB	M	N.D.	23	6	160	3	3
VC	F	4,6	15	3	40	1	0
JD	M	6,1	34	25	10	3	1
DR	F	3,4	14	42	320	2	1
JG	M	3,3	12	8	40	1	0
BR	M	3,3	5	9	0	1	0
KR	F	4,X	34	14	10	3	2
JT	F	4,6	7	10	N.D.	1	1

¹ Age of IDDM onset expressed as months² The time interval between sera drawn and IDDM onset expressed as months³ 1 = lowest; 2 = medium; and 3 = highest band intensities

N.D. - not determined

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As shown in Table 6, 9 out of 12 specimens (75%) were immunoreactive with ^{35}S -GAD₆₅. In addition, two patients (JA and VC) were immunoreactive to GAD₆₇, but not GAD₆₅ under these conditions. Therefore, in combination, autoantibodies to GAD₆₅ and GAD₆₇ were present in 11 out of 12 (91%) of these patients sera. This finding suggests that although autoantibodies to GAD₆₅ are more common than autoantibodies to GAD₆₇, the use of both recombinant GADs (GAD₆₅ and GAD₆₇) in an assay would allow for greater predictability of IDDM. Previous tests of these sera (Atkinson, et al., *Langet*, 335:1357-1360, 1990) demonstrated that 11 out of 12, or 92%, immunoreacted with the ^{35}S -64kD molecule from human pancreatic islet cells. The serum which contained detectable autoantibodies to the 64kD molecule and not GAD₆₅ was a serum which contained the lowest titer (or "1") for the 64kD molecule. Thus, the false negative obtained was due to a lack of sensitivity in this assay. Furthermore, this assay predicted IDDM in one patient (BR) who was negative for 64K.

These results show that the 64kD molecule identified in β -cells of human pancreas is identical in size and antigenicity to rat GAD₆₅. Furthermore, sera drawn from patients prior to IDDM onset contain autoantibodies to GAD₆₅. Consequently, the GAD₆₅ recombinant molecule is of great utility as a diagnostic tool for predicting IDDM. The ability of a physician to diagnose IDDM prior to actual symptoms may result in a greater extension of time before insulin therapy is needed. The sensitivity of such immunoassays will improve with the use of a recombinant GAD₆₅ of human origin which represents the GAD form present in β -cells of the pancreas.

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EXAMPLE 4

IMMUNE PROLIFERATIVE RESPONSE TO POLYPEPTIDE

Polypeptides were synthesized using an automatic instrument (Applied Biosystems) and standard conditions. These polypeptides were then tested to compare their relative ability to stimulate proliferation of splenic lymphocytes and islet infiltrating T lymphocytes (IITLs). In this study, polypeptides derived from the GAD₆₅ core sequence and from the homologous region of polio virus were compared. Appropriate cells were cultured for 5 days with the respective polypeptide in the presence of 5 X 10⁴ irradiated spleen cells. ³H-thymidine was added during the last 16 hours of culture.

15

TABLE 7

ANTIGEN	AMINO ACID SEQUENCE	³ H-THYMIDINE INCORPORATION (cpm)	
		BY LYMPHOID CELL POPULATION 'TILs'	SPLEEN ^b
None	---	1,100	6,500
Poliovirus	MKSMICPQAAQLKVKYL	900	22,500
GAD ₆₅	ARFKMFPEVKEKGMAA	9,500	23,300

• Islet infiltrating T lymphocytes (3×10^4 cells/well)

^b 1×10^6 cells/well

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In these studies, there was no significant difference in the proliferative activity of cultures of spleen lymphocytes exposed to either the polio or the GAD₆₅ polypeptides. However, both polypeptides 5 stimulated a T cell response which was higher than that found in the media control. The lack of difference in proliferation in the spleen cell population may be due to a lower frequency of GAD polypeptide specific T cells.

10 The IITL population, when evaluated in the same manner, showed a marked difference in cell proliferation. In this system, the response to the GAD₆₅ polypeptide was 9-fold greater than that of either the culture media or the polio polypeptide. This data 15 strongly suggests that the GAD₆₅ is an important antigen for T cell responses in the IITL population. This data suggests that molecular mimicry plays a role in the pathogenesis of diabetes.

20

EXAMPLE 5

GAD INDUCES PROLIFERATION OF SPLEEN CELLS OF NOD MICE

Proliferative T-cell responses to β -cell antigens (β CA) develop spontaneously in the nonobese diabetic (NOD) mouse model in a defined chronological order. 25 The NOD mouse experimental model is considered the most analogous *in vivo* system available for studying IDDM in humans. This example describes studies on the antigen-induced blastogenesis of spleen cells from newborn to 5 month old female NOD mice when exposed to GAD and other 30 peptides.

The β CAs tested included one of the two forms of GAD (Kaufman, et al., *Science*, 232:1138-1140, 1986; Erlander, et al., *Neuron*, 7:91-100, 1991; Kaufman, et al., *Trends in Pharm. Sci. (in press)*), (GAD₆₅, 35 previously known as the 64K autoantigen (Baekkeskov, et al., *Nature*, 298: 167-169, 1981; Baekkeskov, et al., *Nature*, 347:151-156, 1990), carboxypeptidase H (CPH)

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(Castano, et al., *J. Clin. Endocrinol. Metab.*, 73:1197-1201, 1991), insulin (Palmer, Predicting IDDM, *Diabetes Reviews*, 1:104-115, 1993) and a peptide of hsp which has been shown to be the immunodominant

5 determinant recognized by NOD T-cells (Elias, *Proc. Natl. Acad. Sci.*, 88:3088-3091, 1991). GAD in particular, is a good candidate for the initial target antigen in IDDM since autoantibodies to GAD arise early in the natural history of the disease (Baekkeskov, 10 *supra*; Atkinson, et al., *Lancet*, 335:1357-1360, 1990; Kaufman, et al., *J. Clin. Invest.*, 89:283-292, 1992). Furthermore, unlike the ubiquitous hsp, GAD is expressed primarily in β -cells and the immunologically privileged central nervous system (CNS) and gonads. As 15 control antigens, irrelevant prototype foreign and self antigens including hen eggwhite lysozyme (HEL), human serum albumin (HSA), *E. coli*. β -galactosidase (β -gal) and murine myelin basic protein (MBP) were used.

NOD (Taconic farms) and BALB/c mice (Jackson 20 Laboratories) were kept under specific pathogen free conditions. The mice were sacrificed at the ages indicated and the spleen cells were tested directly *ex vivo* for their proliferative recall response to antigen. Single cell suspensions of spleen cells were 25 plated at 1×10^6 cells per well in 96 well microtiter plates in 200 μ l serum free HL-1 medium (Ventrex) that was supplemented with 2mM glutamine with or without 10 μ g/ml antigen (or 7 μ M peptide) in triplicate cultures. During the last 16h of the 72h culture period, 1 30 μ Ci [3 H]-thymidine was added per well. Incorporation of label was measured by liquid scintillation counting.

Both human GAD₆₅ (Bu, et al., *Proc. Natl. Acad. Sci.*, 89:2115-2119, 1992) and *E. coli* β -gal (control) were purified from recombinant bacteria on the basis of 35 a hexahistidine tag which allows their rapid affinity purification by metal affinity chromatography (Hochuli, et al., *Bio/Technology*, 6:1321-1325, 1988). Bovine CPH

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was the generous gift of L. Fricker (Albert Einstein Col. Med.) and human insulin was purchased from Eli Lilly.

As illustrated in Figure 8, while proliferative T-cell responses were not detected at any time point to the control antigens, a response to GAD arose at 4 weeks of age in NOD mice, concurrent with the onset of insulitis in the colony. The blastogenesis induced by GAD increased during the next four weeks and then declined to background levels by week 16. At 6 weeks of age, near the peak of anti-GAD reactivity, T-cell responses to hsp appeared and increased until week 15 and then diminished as well (Figure 8). In all NOD mice tested, hsp reactivity was preceded by an anti-GAD response, suggesting that the former reactivity developed as a secondary event during the autoimmune process. Similarly, while no response was detected to CPH at 4 weeks of age, a strong anti-CPH response was observed by week 8. In some mice, a weak response to insulin was observed at 12 weeks, which became more prevalent at 15 weeks of age (Figure 8 and Table 8). None of the antigens induced proliferation in T-cells from age-matched control BALB/c or (NOD x BALB/c) F₁ mice, both of which do not develop insulitis or IDDM. T-cell reactivity subsequently arises to other β CAs, consistent with the inter-molecular diversification of the autoimmune response. Thus, the autoimmune response to GAD was the first to occur among the autoantigens tested. In view of this, tolerization to GAD should prevent the spread of autoimmunity to other β CAs and insulitis. If this were not the case then tolerization to GAD should have no effect on the response to these other antigens.

Blastogenesis provides an approximation of the relative clonal sizes of antigen-specific CD4+ T-cells (Corradin, et al., *J. Immunol.*, 119:1048-1053, 1977). The data in Figure 8 shows that GAD reactive T-cells

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"spontaneously" undergo clonal expansion concurrent with the onset of insulitis. These findings are consistent with an endogenous priming event.

EXAMPLE 6

5

INDUCTION OF TOLERANCE WITH GAD

This example describes a study which shows that induced tolerance to GAD can ameliorate IDDM.

1. In these experiments female NOD mice were intravenously injected at 3 weeks of age with 50 µg GAD, β-galactosidase, mycobacterial hsp65 (m-hsp) or 0.1 µg of the immunodominant hsp peptide (hsp-p), in PBS. At 12 weeks of age, mice were examined for insulitis and autoantigen reactive T-cells. At this age both indications are established in untreated NOD mice.
- 10 15 Pancreatic tissue sections were stained by immunoperoxidase techniques for insulin and were counterstained with hematoxylin. Insulitis was scored in a blinded manner by examining 54 to 87 islets on 5 interrupted tissue sections from each pancreas.
- 20 Proliferative splenic T-cell responses induced by various antigens were performed as described above in Example 4. Data in Table 8 are expressed as the average [³H]-thymidine label (cpm) incorporated in triplicate cultures.

25

TABLE 8A
GAD Induced Tolerance

Treatment	Insulitis Score ^c	N	Spleen Cell Proliferation (SI±SEM) ^b					
			β-Gal	GAD	GAD Peptides		hsp Peptide	CPH
					#17	#34	#35	
Uninjected	2.4±0.2	5	1.0±0.2	<u>9.5</u> ±2.1	<u>4.8</u> ±0.4	<u>8.0</u> ±0.1	<u>2.9</u> ±0.2	<u>8.7</u> ±1.0
β-Gal.	2.6±0.6	5	1.1±0.1	<u>15.4</u> ±1.8	<u>5.1</u> ±0.6	<u>5.1</u> ±0.6	<u>4.0</u> ±0.2	<u>6.6</u> ±0.5
GAD 1.1±0.02	0.1±0.1	8	1.1±0.03	1.6±0.3	1.0±0.05	1.2±0.1	1.0±0.1	1.2±0.1
hsp-p	1.7±0.4	5	1.1±0.05	<u>5.8</u> ±0.2	<u>4.5</u> ±0.1	<u>4.1</u> ±0.3	<u>4.2</u> ±0.1	1.1±0.04
m-hsp	1.8±0.5	5	1.0±0.1	<u>4.2</u> ±0.1	<u>3.9</u> ±0.1	<u>3.9</u> ±0.1	<u>3.4</u> ±0.2	1.0±0.03

* Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = (25%; 2 = 25-50%; 3 = 50-75%; 4 = 75%) (Qin, et al., *Immunolet.*, 150:2072-2080, 1993). Score is mean ± SE.

^b Significant responses noted by solid underline, borderline responses noted by double underline.

^c Not determined.

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TABLE 8B
GAD Induced Tolerance

Treatment	Insulitis Score ^c	N	Spleen Cell Proliferation (SI ± SEM) ^b						
			β -Gal	GAD	#17	GAD Peptides	#34	#35	hsp Peptide
11 peptide	~2.5		1.0 ± 0.1	<u>21.1</u> ± 2.2	<u>13.7</u> ± 1.5	<u>11.4</u> ± 1.5	<u>11.3</u> ± 0.7	<u>13.3</u> ± 0.9	ND
34/35 peptides (+ IFA)	0.7 ± 0.4		1.0 ± 0.2	1.9 ± 1.1	2.2 ± 1.2	1.1 ± 0.3	1.0 ± 0.1	1.8 ± 1.1	ND
IFA alone (+ IFA)	~2.5		1.0 ± 0.1	<u>8.1</u> ± 0.5	<u>5.0</u> ± 0.5	<u>4.8</u> ± 0.5	<u>4.8</u> ± 0.4	<u>6.0</u> ± 0.6	ND

• Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = (25%; 2 = 26-50%; 3 = 50-75%; 4 = 75%) (Qin, et al., *Immuno.*, 150:2072-2080, 1993). Score is mean ± SE.

• Significant responses noted by solid underline, borderline responses noted by double underline.

○ Not determined.

TABLE 8C
GAD Induced Tolerance

Treatment	Insulitis Score^a	N	Spleen Cell Proliferation (SI ± SEM)^b					
			β-Gal	GAD	#17	GAP Peptides #34	#35	hsp Peptide
11 peptide	~(7)		1.4 ± 0.4	<u>12.1</u> ± 3.5	<u>6.9</u> ± 0.4	<u>6.4</u> ± 1.0	<u>6.8</u> ± 0.3	<u>8.7</u> ± 0.9
HEL peptide	(7)		1.2 ± 0.2	<u>10.7</u> ± 3.5	<u>6.4</u> ± 1.4	<u>5.9</u> ± 1.6	<u>6.9</u> ± 1.9	<u>8.0</u> ± 1.8
34/35 peptides (+IFA)	(7)		1.0 ± 0.1	<u>4.4</u> ± 2.6	<u>1.8</u> ± 1.1	<u>1.0</u> ± 0.1	<u>1.1</u> ± 0.2	<u>2.7</u> ± 1.3

^a Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = (25%; 2 = 25-50%; 3 = 50-75%; 4 =) 75%) (Qin, et al., *Immunol.*, **150**:2072-2080, 1993). Score is mean ± SE.

^b Significant responses noted by solid underline, borderline responses noted by double underline.

c Not determined.

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Seventy five percent of the GAD treated mice, but none of the controls, showed no T-cell reactivity to GAD (indicating complete tolerization) or to other β CAs. These mice were also completely free of insulitis (score 0.0). If there were another effector T cell population in the islets, specific for an unknown β CA, that preceded the anti-GAD response, the release of cytokines by this population should have promoted T-cell responses to β CAs and insulitis (Sarvetnick, et al., *Nature*, 346:844, 1990; Heath, et al., *Nature*, 359:547, 1992). Twenty five percent of the GAD-treated mice were not completely tolerized to GAD, as evidenced by a weak residual GAD reactivity (SI of about 3) and displayed very limited peri-insulitis. In contrast, while tolerization to both of the hsp antigens was complete, these treatments reduced, but did not prevent, the development of T cell responses to other β CAs or insulitis. Thus, while the inactivation of GAD-reactive T cells prevented β cell autoimmunity, hsp tolerization only partially reduced it, as would be expected if a secondary element was removed from the amplifactory cascade.

In ongoing experiments examining the effects of GAD tolerization on diabetes incidence, all of the GAD treated mice (n=17, presently 37 weeks old) have normal glucose levels, while 70% of the mice receiving control antigens developed hyperglycemia by 19 weeks of age (n=20). Five GAD treated mice were sacrificed at 30 weeks of age. All were free of detectable β CA reactive T cells. Of these five animals, four mice were completely free of insulitis and one mouse displayed very limited peri-insulitis. These data show that inactivation of GAD reactive T-cells prevents the long term development of insulitis and diabetes.

2. In a second set of experiments neonatal female NOD mice were injected intraperitoneally IFA with peptide 11, a mixture of peptides 34 and 35 plus IFA,

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or with IFA alone and at 12 weeks of age the mice were examined for insulitis and autoantigen reactive T-cells as in Example 6.1. Proliferative splenic T-cell responses induced by the various antigens were 5 performed as in Example 4, and data in Table 8B are expressed as the average [³H]-thymidine label (cpm) incorporated in triplicate cultures.

The data in Table 8B show that tolerization with control peptide 11 did not prevent auto antibody 10 response to GAD or to GAD peptides 17, 34 or 35. Nor was response to hsp peptide prevented by tolerization with peptide 11. IFA alone was somewhat effective at suppressing immune response. By contrast, tolerization with the mixture of peptides 34 and 35 suppressed the 15 autoimmune response of spleen cell proliferation to all species tested: β -gal, GAD, GAD peptides 17, 34 and 35, and hsp peptide. In addition, tolerization to GAD peptides 34 and 35 greatly reduces insulitis but does not completely prevent it as whole GAD65 does.

20 3. In a third set of experiments female NOD mice were intravenously injected at three weeks of age with peptide 11 (control), a mixture of peptides 34 and 35 plus IFA, or with HEL peptides and at 12 weeks of age the mice were examined for insulitis and autoantigen 25 reactive T-cells as in Example 6.1. Proliferative splenic T-cell responses induced by the various antigens were performed as in Example 4, and data in Table 8C are expressed as the average [³H]-thymidine label (cpm) incorporated in triplicate cultures.

30 The data in Table 8C show that tolerization with control peptide 11 did not prevent auto antibody response to GAD, to GAD peptides 17, 34 or 35 or to hsp although the response was not as great as in Example 6.2. Nor was response to hsp peptide prevented by 35 immunization with peptide 11. By contrast, immunization with the mixture of peptides 34 and 35 plus IFA suppressed the autoimmune response of spleen

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cell proliferation to all species tested: β -gal, GAD, GAD peptides 17, 34 and 35, and hsp peptide.

EXAMPLE 7

CHARACTERIZATION OF GAD-REACTIVE T-CELLS

5 This example describes studies on GAD-Reactive T-Cells for additional properties that distinguish activated/memory from resting/naive lymphocytes.

10 In one series of experiments γ interferon (IFN γ) was measured by ELISA in culture supernatants (CSN) of spleen cells of 6-9 week old mice after challenge with GAD or control antigens HEL and MBP. Additionally, the frequency of antigen specific, IFN γ -producing cells was determined by an ELISA spot technique (T. Taguchi, et al., *J. Immunol.*, 145:68-77, 1990). Frequency of 15 antigen-induced, spot forming cells (SFC) among 10^3 spleen cells is represented in Figure 9(a). Values are the mean + SEM from 5 individual female NOD mice, each tested in triplicate cultures with or without antigen. Results from a single experiment are shown. These are 20 representative of 3 separate experiments.

25 In performing these experiments, freshly isolated spleen cells were cultured with or without antigen as described in Example 4. CSN were taken after 48 h and the concentration of IFN γ was determined by ELISA (Macy, et al., *FASEB J.*, 3003-3009, 1988). IFN γ specific monoclonal antibody (mAb) R4-6A2 (Pharmingen) was used as the capturing reagent and biotinylated mAb XMG 1.2 (Pharmingen, also specific for IFN γ) was used 30 in conjunction with streptavidin-alkaline phosphatase (Zymed) and p-nitrophenol for detection of bound lymphokine. Recombinant murine IFN γ (Pharmingen) was used as a standard. ELISA spot assays for the detection of antigen-specific, IFN γ -producing cells were performed as described (Taguchi, et al., *J. Immunol.*, 145:68-77, 1990). After a 24h pre-activation culture of spleen cells with our without antigen, cells 35 were transferred by serial dilution to 96 well

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microtiter plates (Millipore) that had been pre-coated with mAb R4-6A2. After 24h, the cells were removed and IFN γ spots were visualized using XMG 1.2-biotin in conjunction with nitroblue terazolium-

5 bromochloroindolyl phosphate substrate (Sigma). Spots were counted visually and the frequency of antigen specific cells was determined from the difference between the number of spots seen with and without antigen.

10 As shown in Figure 9(a), when freshly isolated T-cells from 6-9 week old NOD mice were challenged with GAD or control antigens, high concentrations of IFN γ were detected only in cultures containing GAD, suggesting that the GAD specific T-cells had been pre-activated in vivo, since only pre-activated T-cells

15 (Th1) produce IFN γ within 48 hours after antigen recognition (Ehlers, et al., *J. Exp. Med.*, 173:25-36 1991; Croft, et al., *J. Exp. Med.*, 176:1431-1437, 1992). In contrast, T-cells from age matched BALB/c

20 mice did not respond to GAD or to control antigens by IFN γ production (data not shown).

Results of the ELISA spot assay to measure directly the frequency of GAD-specific T-cells showed that while in 6-9 week old NOD mice, T-cells reactive to control antigens constituted approximately 1 in 10^5 cells in the spleen, the frequency of GAD-reactive T-cells was about two orders of magnitude higher, ranging from 90-291 cells per 10^5 cells (Figure 9(a), confirming the data obtained by proliferation assays (Figure 8) that these 30 cells had been clonally expanded in vivo.

In another series of experiments, GAD specific T-cells were characterized for expression of the cell surface marker L-selectin, since murine T-cells convert from an L-selectin $^+$ (L-sel $^+$) to an L-selectin $^-$ (L-sel $^-$) phenotype upon activation (Bradley, et al., *J. Immunol.*, 148:324-331, 1992).

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To perform these studies, pooled spleen cells from 3 to 4 age matched mice were panned on plates coated with goat-anti-mouse Ig (Zymed) to remove adherent macrophages as well as B cells. Next, CD8+ cells were 5 coated with mAb 58.6-72 (ATCC) and removed by panning over plates coated with goat-anti-rat Ig (Zymed). The non-adherent CD4+ cell fraction was labeled with anti-L-selectin mAb MEL-14 (ATCC) and panned on goat-anti-rat Ig coated plates. Both the adherent (CD4+ L-sel⁺) 10 and non-adherent (CD4+,L-sel⁻) fractions were sampled. Purity of the cell fraction was assessed by FACS analysis; cells were >90% CD4+ and >95% enriched for the L-sel⁻ or L-sel⁺ phenotype. The purified cell 15 fractions were tested for GAD reactivity by seeding them at 2 x 10⁵ cells per well in 96 well microtiter plates with or without antigen. Irradiated (3000 rad), unseparated spleen cells of 3 week old NOD mice were added at 5 x 10⁵ cells per well as a source of antigen presenting cells. Supernatants of triplicate cultures 20 were taken 48h later and their IFN γ content was determined by ELISA.

The results of this study showed that by 2-3 weeks of age, GAD reactive T-cells could not be detected in either the L-sel⁺ or the L-sel⁻ population, consistent 25 with a low frequency of antigen reactive precursors at this time point. However, by 6 weeks of age high levels of IFN γ were induced by GAD (but not by control antigens) in the L-sel⁻ (but not the L-sel⁺) subpopulation of CD4+ cells (Figure 9(b)).

30 The increase in clonal size of GAD reactive T-cells, their production of IFN γ and their L-sel⁻ phenotype provide three independent lines of evidence that a potentially pathogenic (Ando, et al. *Cell Immunol.*, 124:132-143, 1989) Th1 type T-cell response 35 is spontaneously primed to GAD *in vivo* early in NOD development.

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EXAMPLE 8

CHARACTERIZATION OF GAD SPECIFIC T-CELL

DETERMINANT RECOGNITION

The fine specificity of the anti-GAD T-cell
 5 response was mapped using a set of 38 peptides
 (numbered successively from the N-terminus) that were
 20-23 amino acids (aa) long and span the entire GAD₆₅
 (Bu, et al., Proc. Natl. Acad. Sci., 89:2115-2119,
 1992) sequence with 5aa overlaps (Figure 10).

10 Spleen cells were tested from 4 (Figure 10a), 5
 (Figure 10b) and 7 (Figure 10c) week old NOD mice for
 proliferative responses (as described in Example 4) to
 the GAD peptides. Peptides were present in cultures at
 7 µM and the label was added during the last 16 hours
 15 of a 5-day culture. The peptides were synthesized
 using standard Fmoc chemistry and purified by reverse
 phase HPLC (Advanced Chemtech). The sequence of
 stimulatory peptides are shown below in Table 9.

TABLE 9

20

	<u>Peptide Number</u>	<u>GAD Region</u>	<u>Amino Acid</u>
	<u>sequence</u>		
	6	78-97	
	KPC <u>SCSKVDVNYAFLHATDL</u>		
25	17	247-266	NMYAM <u>M</u> IAR <u>F</u> KMFPEVKEKG
	23	335-356	TAGTTVYGA <u>F</u> DPLLAVADICKK
	32	479-498	EYLY <u>N</u> IKNREGYEMVFDGK
	34	509-528	<u>I</u> PPSLRYLEDNEERMSRLSK
	35	524-543	SRLSKVAPVIKARMMEYGTT
30	36	539-558	EYGTTMVS <u>Y</u> QPLGDKVNFFR
	38	566-585	ATHQDIDFLIEEIERLGQDL

Murine and human GAD₆₅ are 95% identical at the
 35 amino acid level (555/585) and are 98% conserved, with
 most of the differences localized near their N-termini.
 The underlined amino acid in the stimulatory peptide

sequences above are conservatively substituted in murine GAD₆₅. In separate experiments, the murine form of key peptides (#17 and #34) were tested and produced similar results.

As shown in Figure 10, peptides that triggered stimulation indices ≥ 3 are indicated as black bars. These peptides did not induce proliferation in T-cells from NOD mice (≤ 3 or ≥ 16 weeks in age, or from control (BALB/c x NOD)F1 mice (data not shown). The data are represented as the mean SI \pm standard error calculated from 3-6 individual mice tested twice in each age group. Characteristic results for peptide induced blastogenesis in individual mice are shown in Table 6. The first detectable response, at 4 weeks of age, was confined to the carboxy-terminal region of GAD, and involved two adjacent peptides (aa 509-528 and 524-543, peptides #34 and #35, respectively, Figure 10a). At 5 weeks of age, responses to an additional determinant (aa 247-266, peptide #17, which contains a region of sequence similarity with Coxsackievirus (Kaufman, et al., *J. Clin. Invest.*, 89:283-292, 1992) (Figure 10b) were regularly recorded. During the next two weeks, responses to peptide #17 (aa 247-266) increased and T-cell autoimmunity spread to two additional peptides at the carboxy terminus (aa 479-498 and 539-558; peptides #32 and #36 respectively, Figure 10c). Subsequently, reactivity to the GAD peptides declined (data not shown), paralleling the loss of response to the whole protein (Figure 8). It is unclear why the initial T-cell response to β CAs fades in NOD mice. Possible explanations include: a) immune regulatory mechanisms; b) exhaustion of the response due to the continuous stimulation by the endogenous antigen; and c) induction of anergy in specific T-cells owing to their recognition of the autoantigen on "non professional" antigen presenting cells such as the β cells themselves (Markmann, et al., *Nature*, 336:476-479, 1988).

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The gradual diversification of the primed autoreactive T-cell repertoire that was observed in this naturally occurring autoimmune disease parallels the shifts in T-cell recognition recently observed in 5 experimentally induced autoimmunity to the CNS where autoreactivity spreads both intra- and intermolecularly among CNS proteins (Lehmannn, et al., *Nature*, 358:155-157, 1992; Perry, et al., *J. Neuroimmunol.*, 33:7-15, 1991; Watanabe, et al., *Nature*, 305:150-153, 1983; 10 Liebert, et al., *J. Neuroimmunol.*, 17:103-118, 1988). Apparently, lymphokine secretion by the first wave of autoantigen specific T-cells in the target organ results in up-regulation of antigen presentation and creates a microenvironment that favors priming of 15 additional autoreactive T-cells (Lehmann, et al., *Immunol. Today*, 14:203-208, 1993; Sarvetnick, et al., *Nature*, 346:844-847, 1990; Heath, et al., *Nature*, 359:547-549, 1992). Since hsp reactive CD4+ T-cells are capable of inducing IDDM (Elias, et al., *Proc. 20 Natl. Acad. Sci.*, 87:1576-1580, 1990; Elias, et al., *Proc. Natl. Acad. Sci.*, 88:3088-3091, 1991), their recruitment into the activated T-cell pool, along with T-cells reactive to other β CAs, probably reflects an amplificatory cascade that eventually leads to β cell 25 destruction.

In summary, the data above establish GAD as a critical target antigen in the pathogenesis of IDDM in NOD mice. The results show that T-cell responses to β CAs diversify both intramolecularly and 30 intermolecularly as the disease progresses, consistent with a dynamic autoimmune repertoire (Lehmann, et al., *Immunol. Today*, 14:203-208, 1993). However, interference with the early autoreactive T-cell population can prevent the recruitment of additional 35 autoantigens into the primed repertoire thereby halting a cascade of autoimmune responses that eventually leads to β cell destruction. As a similar autoimmune

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progression is also likely to occur during the development of human IDDM (Palmer, J.P., Predicting IDDM, *Diabetes Reviews*, 1:14-115, 1993; Atkinson, et al., *Lancet*, 339:458-459, 1992), these findings suggest
5 that peptide-based immunotherapeutic agents would be useful in predicting and ameliorating human IDDM.

EXAMPLE 9

AUTOANTIBODY REACTIVITY WITH GAD FRAGMENTS

This example describes a study which examined the
10 variability in recognition of epitopes in human GAD₆₅ polypeptides by IDDM autoantibodies in sera of human patients.

Portions of human GAD₆₅ cDNA were amplified by the polymerase chain reaction (PCR; Saiki, et al., *Science*, 15 239:487, 1988) to produce DNA segments encoding three polypeptide segments: amino acid residues 1-224 (segment A); 224-398 (segment B); and 398-585 (segment C). Each construct also contained a T₇ promoter, a consensus sequence for the initiation of translation
20 and an initiating methionine codon (Korak, M., *J. Cell Biol.*, 108:229, 1989). Each PCR product was then transcribed in vitro with T₇ RNA polymerase and translated in vitro in a rabbit reticulocyte cell-free system in the presence of ³⁵S-methionine, using
25 conditions recommended by the supplier (Amersham Corp., Arlington Heights, IL). Each test serum (30 µl) was incubated with the resulting ³⁵S labeled-polypeptides. The bound peptides were isolated with PAS and analyzed by SDS-PAGE in 12% polyacrylamide and autoradiography.

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TABLE 10
IDDM PATIENT SERA REACTIVITY WITH GAD SEGMENTS

5	PATIENT	SEGMENT		
		A	B	C
	Control (N=7)	-	-	-
	052	-	+	+
	723	-	-	-
	705	-	+	+
10	UC2	-	+	+
	N.L.	-	-	-
	L.I.	-	-	-
	T.L.	-	-	-
	P.T.	-	+	-
15	J.D.	-	-	-
	B.Y.	-	+	+
	M.C.	-	-	-
	R.S.	-	-	-
	K.O.	-	-	-
20	T.B.	-	-	-
	S.M.	-	-	-
	A.W.	-	+	-
	J.B.	-	+	+
	J.A.	-	-	-
25	P.C.	-	+	+
	L.R.	-	-	-
	J.M.	-	+	-
	G.A.	-	-	-

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As shown in Table 10, none of the specimens had detectable levels of antibodies to the amino terminal third (segment A) of GAD whereas 9 patients (41%) had antibodies reactive with the middle third (segment B) 5 and 6 patients (27%) had antibodies to the carboxyl-terminal third (segment C) of GAD.

EXAMPLE 10

PREDICTION OF INCIPIENT IDDM BY GAD

EPITOPE RECOGNITION PATTERN

10 The increasing likelihood of an IDDM interventive therapy and the (recently acknowledged) benefits of managed glucose homeostasis in preventing IDDM associated complications makes the early detection of β cell autoimmunity before clinical IDDM onset and in 15 NIDDM patients (10% of whom eventually convert to IDDM) a crucial goal. Autoantibodies to GAD may provide the earliest and most reliable marker of impending IDDM among the molecularly defined IDDM associated 20 autoantigens. To determine whether GAD peptides will bind to IDDM associated autoantibodies the following study was conducted.

A set of peptides (20-23 amino acids in length, with 5 aa overlaps) that span the human GAD65 molecule were synthesized to determine whether sera from most 25 individuals at risk, pre-IDDM and with IDDM (in contrast to healthy controls) do in fact produce antibodies that differentially recognize GAD65 linear epitopes distributed throughout the molecule.

Patient sera and most control sera were those used 30 in a previous study (Kaufman, et al., J. Clin. Investigation, *supra*) All samples were coded and tested in a blind manner. Peptides were synthesized using an automatic instrument (Applied Biosystems, Foster City, CA) and standard conditions. Peptides were dissolved 35 in 60 mM sodium bicarbonate buffer (pH 9.6) at 20 ug/ml and 100 ul of each was added to duplicated wells of a 96 well Nunc-Immuno Plate. Peptides were allowed to

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bind at 4°C overnight. The plates were then washed three times with PBS + 0.1% Tween 20 (wash buffer), after which the plates were pre-absorbed with 3% BSA in sodium bicarbonate buffer for 0.5 hours at 37°C, or at room temperature overnight. The plates were then washed 5 times with the above wash buffer. 100 ul of serum at a 1/300 dilution in PBS + 0.1% Tween 20 and 1% BSA was added to each well and antibodies were allowed to bind for 1 hour at 37°C. The plates were washed 5 times with wash buffer. 100 ul of a 1/600 dilution of HRP-goat anti-human IgG (BRL, Gaithersberg, MD) was added to each well and allowed to bind for 1 hour at 37°C. The plates were then washed 7 times and 100 ul of substrate buffer was added to each well for 30 minutes at room temperature. The color development was measured at 410 nm using an ELISA plate reader (ICN, Biomedicals, Costa Mesa, CA). Positive sera were defined as: OD₄₁₀ of the sample/negative control ≥3.0.

The data shown in Table 11 establish that a number of GAD peptides were recognized by patients previously shown to be 64K positive, but not by control sera. Each patient showed a different pattern of GAD epitope recognition. Peptides 20, 21 and 25, were each recognized by 6/8 patients, and none of the controls--with the exception of peptide 25 which was recognized by 1 out of 13 controls. Based on immunoreactivity to 2 of these peptides (#20 and 21) 7/8 (88%) of the patients (and none of the controls) could be identified as possessing GAD autoantibodies. Peptides 3, 6, 22, 25 and 37 were each recognized by only 25-37% of the patients (and none of the control sera), but taken together, 75% of the patients recognized at least one of these. Peptides 5, 9 and 24 were often positive for immunoreactivity by both control and patient sera.

This level of sensitivity is comparable to the best currently available assays using whole GAD65 purified

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from brain or recombinant organisms. Besides avoiding laborious antigen purification, peptide based autoantibody screening, together with PCR based HLA typing, may reveal epitope recognition patterns 5 associated with progression or lack of progression to IDDM and its associated complications. Individuals determined to be at high risk could then consider therapeutic intervention.

It should also be noted that the GAD peptides 10 recognized by autoantibodies were different from those recognized by NOD GAD reactive T cells in Example 6.

TABLE 11
EPITOPE RECOGNITION OF HUMAN GAD65 PEPTIDES

	PEPTIDE																																					
5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Pre-IDDM																																						
J.A.										x	x																											
723										x	x																											
P.T.	(1)	x	x	x	x	b	b	b	b																													
	(2)	10	b	x	b	b	b	b	b																													
J.B.	(1)		b	b	b	b	b	b	b																													
	(2)	b	x	b	b	b	b	b	b																													
R.S.	(1)	x	x	b	b	b	b	b	b																													
	(2)	x	x	b	b	b	b	b	b																													
B.Y.	(1)	15	x	x	b	b	b	b	b	x																												
	(2)	x	x	b	b	b	b	b	b	x																												
J.B.	(1)		b	x	b	b	b	b	b	b																												
	(2)	b	x	b	b	b	b	b	b	b																												
At Onset	20									x	x																											
705										x	x																											
S.H.	b	x	x	x	x	b																																
291	b	x	x	x	x	b																																
048	x	x	x	x	x	x																																
25	5	1	2	2	3	2																																

X = positive for immunoreactivity as defined by OD/background>3.0
 30b = borderline response (OD/background >2.5-2.9).

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EXAMPLE 11

GAD IMMUNIZATION PROTECTS NOD MICE FROM IDDM

The availability of cDNAs encoding GAD65 allows the testing of this molecule in new interventive therapies designed to interfere with GAD-specific T cells. Tests were conducted to examine the ability of GAD65 immunization to protect NOD mice at 8 weeks of age, a time at which T cell responses to a number of b cell antigens and insulitis is already well established. If GAD immunotherapy was effective at this stage, it would hold promise for treatment in humans in which the autoimmune process has already been established.

METHODS

Antigens

An IPTG inducible T7 expression vector was used to express both human GAD65 and *E. coli* b galactosidase (β -gal). In IPTG induced recombinant *E. coli*, GAD and β -gal constitute about 10-20% of the total bacterial protein. However, almost all of the GAD was in inclusion bodies, which could be isolated and extensively washed to obtain material that is about 80% GAD. We then did affinity purifications of GAD and β -gal on the basis of a hexa-histidine "tag" which was attached to GAD during the subcloning process. These extra histidine residues allow the rapid affinity purification (Novagen) of GAD by metal affinity chromatography (Hochuli, et al., Bio Technology, 6:1321-1325, 1988). The inclusion body material is solubilized in 6M guanidine hydrochloride (GHCL), 10mm β -mercaptoethanol and 1% triton X-100. After binding to the column, the column was extensively washed with GHCL and 8M urea in phosphate buffers. Only the central peak GAD fraction was utilized for subsequent studies. Human GAD65 shares 96% amino acid sequence identity with murine GAD65, with most of the amino acid differences being conservative substitutions.

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The GAD preparation appeared to be free of immunologically detectable contaminants. It also appeared to be free of bacterial contaminants on overloaded silver stained gels. Analysis by a national reference laboratory found (0.06ng LPS/ug GAD. Human GAD65 did not induce T cell proliferation in (4 or)16 week old NOD or control BALB/c or (NOD/BALB/c) F1 spleen cells. The results using synthetic GAD peptides (Figure 10) precisely parallel the data using whole recombinant GAD (Figure 8). Other antigens described herein elsewhere that are not involved in IDDM (such as the beta galactosidase) did not induce NOD T cell responses. After immunizing mice with GAD, we were unable to detect cross reactive T cell responses in recall experiments with other proteins that were purified from recombinant *E. coli* by the same metal affinity chromatography procedure. Amino acid sequence analysis of GAD and β -gal each gave a single expected amino acid N-terminal sequence. If there had been appreciable endotoxins, heat shock proteins, or other contaminants present in the GAD preparation, spleen, PBMC (Atkinson, et al.), and T cell proliferation responses that were not disease specific would have been expected.

Breeder mice were purchased from Taconic Farms and housed under specific pathogen-free conditions. Only female NOD mice were used in this study. The average age of IDDM onset in unrelated females in the colony was 22 weeks. Insulitis is generally observed beginning at 4 weeks of age. T cell responses to GAD, HSP, CPH were found by 6 weeks in age. The incidence of IDDM in female mice is 70-90% by one year of age.

Immunizations

At 8 weeks of age, 25 ug GAD or control β -gal. was injected intraperitoneally (ip) in 100 ul of incomplete Freunds adjuvant (IFA). Because there may be a requirement for continual antigen presentation

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(Ramsdell, et al., Science, 257:1130-1133, 1992) mice were treated again every 6 weeks. Urine glucose levels were monitored twice weekly. After observing above normal glucose in urea, blood glucose levels were 5 monitored twice weekly. Two consecutive blood glucose level readings of 300 mg/ml was considered as IDDM onset, after which the mice were sacrificed and spleen cells were tested as described above in Example 6 for evidence of spleen cell proliferation.

10 Immunization of 8 week old NOD mice produced a clear delay in the onset of IDDM compared to control β -gal immunized mice (Fig. 11). While two of the GAD immunized mice (open circles) developed IDDM at about the normal age of onset (20 weeks), the other 8 GAD 15 immunized mice showed no signs of hyperglycemia until 36 weeks in age. Four of the GAD treated mice developed IDDM between 37 and 40 weeks in age. Four of the GAD treated mice currently remain disease free (at 52 weeks of age). In contrast, the majority of β -gal 20 injected mice (closed circles) had hyperglycemia by 22 weeks of age and 6/10 developed IDDM by 27 weeks in age. At 52 weeks of age, 2 of the β -gal treated mice remain disease free. This experiment shows that GAD 25 immunization significantly delayed (<0.02) or prevented diabetes of NOD mice in which β cell autoimmunity has already significantly progressed.

β cell autoimmunity is already well established at 8 weeks of age, and it is likely to also be in 30 individuals determined to be at risk for IDDM on the basis of circulating autoantibodies. Although the mechanism of this protection is not clear, periodic injections of GAD have a profound moderating effect on the induction of disease.

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TABLE 11
AMINO ACID SEQUENCES FOR GAD65

	1	MASPGSGFWSGSEDGSGDS
5	2	GSGDSENPGTARAWCQVAQKFTG
	3	QKFTGGIGIGINKLCALLYGD
	4	LLYGDAEKPAESGGSQPPRA
	5	QPPRAAARKAACACDQKPCSC
	6	KPCSCSKVDVNYAFLHATDL
10	7	HATDLLPACDGERPTLAFLQ
	8	LAFLQDVVMNILLQYVVKSFDRS
	9	SFDRSTKVIDFHYPNELLQE
	10	ELLQEYNWELADQPQNLEEILM
	11	EEILMHQCQTLKYAIKTGHP
15	12	KYGHPRYFNQLSTGLDMVGL
	13	DMVGLAADWLTSTANTNMFT
	14	TNMFTYEIAPVFVLLEYVTL
	15	EYVTLKKMREIIIGWPGGSGD
	16	GGSGDGIFSPGGAIISNMYAM
20	17	NMYAMMIARFKMFPEVKEKG
	18	PEVKEKGMAALPRLIAFTSE
	19	AFTSEHSHFSLKKGAAALGI
	20	AALGIGTDSVILIKCDERGK
	21	DERGKMIPLSDLERRILEAKQ
25	22	LEAKQKGFPFLVSATAGTT
	23	TAGTTVYGAFDPLLAVADICKK
	24	DICKKYKIWMHVDAAWGGLLMS
	25	GLLMSRKHKWKLSGVVERANS
	26	ERANSVTWNPHKMMGVPLQC
30	27	VPLQCSALLVREEGLMQNCNQ
	28	QNCNQMHASYLFQQDKHYDL
	29	KHYDLSYDTGDKALQCGRHV
	30	CGRHVDVFKLWLMWRAKGTG
	31	KGTTGFEAHVDKCLELAEYLYN
35	32	EYLYNIIKNREGYEMVFDGK
	33	VFDGKPQHTNVCFWYIPPSL
	34	IPPSLRTLEDNEERMSRLSK

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35 SRLSKVAPVIKARMMEYGTT
36 EYGTMMVSYQPLGDKVNFFR
37 VNFFRMVISNPAATHQDIDF
38 ATHQDIDFLIEEIERLGQDL

5

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made without departing 10 from the scope of the invention.

CLAIMS

1. A polypeptide consisting essentially of an amino acid sequence selected from the group consisting of:
 - 5 KPCSCSKVDVNYAFLHATDL;
 - NMYAMMIARFKMFPEVKEKG;
 - TAGTTVYGAFDPLLAVADICKK;
 - EYLYNNIIKNREGYEMVFDGK;
 - 10 IPPSLRYLEDNEERMSRLSK;
 - SRLSKVAPVIKARMMEYGT;
 - EYGTTMVSYQPLGDKVNFFR;
 - ATHQDIDFLIEEIERLGQDL;
 - LAFLQDVNMILLQYVVKSFDRS;
 - 15 EEILMHQCQTLKYAIKTGHP;
 - DERGKMIPISKLERRILEAKQ;
 - KHYDLSYDTGDKALQCGRHV;
 - AALGIGTDSVILIKCDERGK;
 - GLLMSRKHKWKLSGVERANS;
 - 20 LEAKQKGFVPFLVSATAGTT; and
 - VNFFRMVISNPAAATHQDIDF.
2. An isolated polynucleotide sequence encoding a polypeptide of claim 1.
3. The polynucleotide of claim 2 which is DNA.
- 25 4. A method for detecting autoantibody to GAD in a patient specimen which comprises contacting the specimen with a GAD fragment and determining whether autoantibody binds to the fragment.
5. The method of claim 4, wherein the fragment is a polypeptide of at least about 16 amino acids in length and is selected from the amino acid sequence from about amino acid 224 to about amino acid 585 of GAD₆₅.
- 30 6. The method of claim 4, wherein the fragment is a polypeptide of claim 1.
7. The method of claim 4, wherein the specimen is blood.

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8. The method of claim 4, wherein the peptide is detectably labeled.
9. The method of claim 8, wherein the detectable label is selected from the group consisting of a radioisotope, a fluorescent compound, a colloidal metal, a chemiluminescent compound, a bioluminescent compound, a phosphorescent compound, and an enzyme.
10. The method of claim 4, wherein the peptide is bound to a solid phase.
11. A monoclonal antibody to the peptide of claim 1.
12. A hybridoma cell line capable of producing the monoclonal antibody of claim 9.
13. A vector containing the polynucleotide of claim 2.
14. A host cell transformed with the polynucleotide of claim 2.
15. A method for ameliorating a GAD associated autoimmune disorder in a patient having or at risk of having the disorder, which comprises administering to the patient a therapeutically effective amount of whole GAD₆₅ or a therapeutically effective fragment thereof.
16. The method of claim 15, wherein the therapeutically effective fragment is a polypeptide of at least about 16 amino acids in length and is selected from the amino acid sequence from about amino acid 224 to about amino acid 585 of GAD₆₅.

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17. The method of claim 15, wherein the therapeutically effective fragment is selected from the group consisting of:

KPCSCSKVDVNYAFLHATDL;
5
NMYAMMIARFKMFPEVKEKG;
TAGTTVYGAFDPLLAVALDICKK;
EYLYNIIKNREGYEMVFDGK;
IPPSLRYLEDNEERMSRLSK;
SRLSKVAPVIKARMMEYGT;
10
EYGTTMVSYQPLGDKVNFFR; and
ATHQDIDFLIEEIERLGQDL.

18. The method of claim 15, wherein the therapeutically effective fragment is selected from the group consisting of:

15
KPCSCSKVDVNYAFLHATDL;
LAFLQDVNMNILLQYVVKSFDRS;
EEILMHQC TTLKYAIKTGHP;
DERGKMI PSKLERRILEAKQ;
KHYDLSYDTGDKALQCGRHV;
20
AALGIGTDSVILI KCD ERGK;
GLLMSRKHKWKL SGVERANS;
LEAKQKG FVPFLVSATAGTT; and
VNFFRMVISNP AATHQDIDF.

19. The method of claim 16, wherein the disorder is
25
IDDM.

20. The method of claim 16, wherein the disorder is
stiff man disease.

21. The method of claim 16, wherein the
administration is enteral.

- 30
22. The method of claim 21, wherein the enteral
administration is oral.

23. The method of claim 15 or 16, wherein the
administration is parenteral.

- 35
24. The method of claim 23, wherein the parenteral
administration is by subcutaneous, intramuscular,
intraperitoneal, intracavity, transdermal,
intranasal, or intravenous injection.

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25. The method of claim 15, wherein said administration is at a dosage of about 0.01 mg/kg/dose to about 2000 mg/kg/dose.
26. The method of claim 15, wherein the polypeptide is therapeutically labeled.
5
27. The method of claim 26, wherein the therapeutic label is selected from the group consisting of a radioisotope, a drug, a lectin, and a toxin.
28. A method for detecting the status of a GAD associated autoimmune disorder in a patient which comprises contacting a T-cell of the patient with at least one polypeptide of claim 1 and detecting the response of the T-cell to the peptide.
10
- 15 29. The method of claim 26, wherein the response of the T-cell is stimulation.
30. The method of claim 27, wherein the stimulation is measured by cellular uptake of radiolabelled nucleoside.
- 20 31. The method of claim 30, wherein the nucleoside is ³H thymidine.
32. A kit useful for the detection of antibody to the polypeptide of claim 1 in a specimen suspected of containing such antibody, the kit comprising carrier means compartmentalized to receive in close confinement therein one or more containers comprising a container containing the polypeptide of claim 1.
25
33. A kit useful for determining the status of a GAD associated disorder in a specimen of a patient, the kit comprising carrier means compartmentalized to receive in close confinement therein one or more containers comprising a container containing a polypeptide of claim 1.
30

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34. A method for detecting a GAD associated autoimmune disorder in a patient having or at risk of having the disorder, which comprises administering to the patient a diagnostically effective amount of a diagnostically effective fragment of GAD₆₅.
- 5 35. The method of claim 34, wherein the diagnostically effective fragment is a polypeptide of at least about 16 amino acids in length and is selected from the amino acid sequence from about amino acid 34 to 573 of GAD₆₅.
- 10 36. The method of claim 35, wherein the diagnostically effective fragment is selected from the group consisting of:
 - 15 KPCSCSKVDVNYAFLHATDL;
 - LAFLQDVVMNILLQYVVKSFDRS;
 - EEILMHQCQTLKYAIKTGHP;
 - DERGKMIPLSKLERRILEAKQ;
 - KHYDLSYDTGDKALQCGRHV;
 - 20 AALGIGTDSVILIKCDERGK;
 - GLLMSRKHKWKLSGVERANS;
 - LEAKQKGFPFLVSATAGTT; and
 - VNFFRMVISNPAAATHQDIDF.
- 25 37. The method of claim 35, wherein the disorder is IDDM.
38. The method of claim 35, wherein the disorder is stiff man disease.

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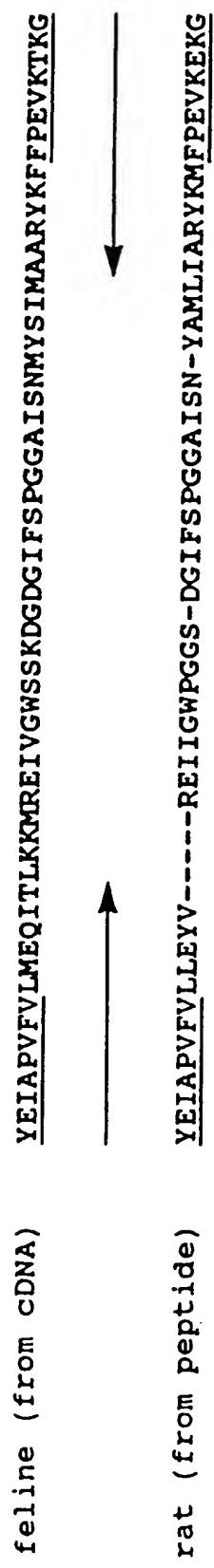


FIG. 1

SUBSTITUTE SHEET (RULE 26)

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GGCGGTGGGGTGCAGCCGAAGCAGCTTGCCCCGAGCCACTCGGAGGGACCGGCCA
10      M A S P G S F W S F G S E D G
GACTAGCAGAACCCATGGCATCTCCGGCTCTGGCTTTGGATCTGAAGATG
70      S G D P E N P G T A R A W C Q V A Q K F
GCTCTGGGATCCTGAGAACCCGGGAACAGCGAGGCTGGTGGCCAGGTGGCCCCAAAGT
90
T G G I G N K L C A L L Y G D S E K P A
TCACGGGGCATCGAAACAAGCTATGGCTCTGGCTTAACGGAGACTCTGAGAAGGCCAG
130
E S G G S V T S R A A T R K V A C T C D
CAGAGCGGGGAGCGGTGACCTCGGGGGCCACTCGGAAGGTGGCCTGCACCTGTG
150
Q K P C S C P K G D V N Y A L L H A T D
ACCAAAACCCTGCAGCCTGGCCAAAGGAGATGTCAATTATGCACTTCTCCACGCCAACAG
190
L L P A C E G E R P T L A F L Q D V M N
ACCTGCTGCCAGCCTGTGAAGGAGAAAGGGCCACTCTCGCATTCTGCAAGATGTAATGA
210
I L L Q Y V V K S F D R S T K V I D F H
ACATTTGCTTCAGTAGCTGGTGAAGGTTGATAGATAACTAAAGTGATGATTCC
250
Y P N E L L Q E Y N W E L A D Q P Q N L
ATTACCCCAATGAGCTTCAAGAGTATAATTGGGAATTGGCAGACCAACGGCAAATC
430
450
490
510
550
570
590
610
630
650
R Y F N Q L S T G L D M V G L A A D W L
TGGAGGAATTGACGCCACTGCAAAACTAAATATGCGATTAAACAGGGCATIC
CCCGATATTAAATCAGCTGTCTACCGGATTGGATATGGTTGAGGAGATTGGT
T S T A N T N M F T Y E I A P V F V L L
TGACATCAACAGCAAACACGAACATGTTACCTATGAGATCGCCCCCTGTATTGTACTAC

```

FIG. 2A

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670 E V T L K M R E I I G W P G G S G D
 TGGAAATATGTGACACTAAAGAAAATGAGGGAAATCATTTGGCTGGCCAGGAGGCTCTGGCG
 730 G I F S P G G A I S N M Y A M L I A R Y
 ATGGAATCTTCTCCTGGTGGCCATCTCCAACATGTACGCCATGGCTCATGGCCGCT
 790 K M F P E V K E K G M A A V P R L I A F
 ATAAGATGTTTCCAGAAGTCAAGGAAAAGGGATGGGGGGATGGCCAGGCTCATGGCAT
 850 T S E H S H F S L K K G A A V P R L I A F
 TCACGTCAGAGCATAGTCACTTCTCAAGGAAGGGCTGGGAGGCTGGCAGGCCATGGGAA
 910 D S V I L I K C D E R G K M I P S D L E
 CAGACAGCGGTGATTCTGATTAAATGATGAGAGAGGGAAATGATCCCCATCTGACCTTG
 970 R R I L E V K Q K G F V P F L V S A T A
 AAAGAAGAACATCCTTGAAGTCAAACAGAAAGGATTGTTCCCTGGTAGCTGCCACAG
 R R I L E V K Q K G F V P F L V S A T A
 AAAGAAGAACATCCTTGAAGTCAAACAGAAAGGATTGTTCCCTGGTAGCTGCCACAG
 1030 G T T V Y G A F D P L L A V A D I C K
 CTGGAAACCACTGTTGACGGGGCTTGTGATCCCTCTGGCTTAGCTGACATCTGCAAA
 1090 Y K I W M H V D A W G G G L L M S R K
 AATAATAAGATCTGGATGGCATGTGGATGGCTGGCTGGGGTTACTGATGTCTGGAA
 1150 H K W K L N G V E R A N S V T W N P H K
 AACACAAAGTGGAAAGCTGAACGGTGTGGAGGGCCAAACTCTGTGACATGGAAATCCCACA
 1210 M M G V P L Q C S A L L V R E E G L M Q
 AGATGATGGGTGCTCCCTTGCATGTTGGCTCCTGGTCAGAGAGGGACTGATGCG

FIG. 2B

SUBSTITUTE SHEET (RULE 26)

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S	C	N	Q	M	H	A	S	Y	L	F	Q	Q	D	K	H	Y	D	L	S	
AGAGCTGCAACCAGATGCATGCTTACCTTCAGCAAGATAAGCAACTATGACCTGT																				
1330																				
Y	D	T	G	D	K	A	L	Q	C	G	R	H	V	D	V	F	K	L	W	
CCTATGACACGGAGACAAGGGCCTTGCACTGTGGACCGCCACGTCGATGTCATTAAATTAT																				
1390																				
L	M	W	R	A	K	G	T	T	G	F	E	A	H	I	D	K	C	L	E	
GGCTCATGGAGAGCAAAGGGCACTACTGGATTITGAAGCTCACATTGATAAGCTGTGTTGG																				
1450																				
L	A	E	Y	L	Y	N	I	I	K	N	R	E	G	Y	E	M	V	F	D	
AGCTGGCAGAGTATTATAAATTACATTAAACCCGAGAACGATATGAAATGGTGTTCG																				
1510																				
G	K	P	Q	H	T	N	V	C	F	W	F	V	P	P	S	L	R	V	L	
ATGGGAAGGCCTCAGCACACAAATGTCTGCTTCTGGTTGTACCTCCTAGTTGGAGTTG																				
1570																				
E	D	N	E	E	R	M	S	R	L	S	K	V	A	P	V	I	K	A	R	
TGGAAAGACAATGAAGAGAAATGAGCCGCCCTCTCAAAGGTGGCCAGTGATTAAAGCCA																				
1630																				
M	M	E	Y	G	T	T	M	V	S	Y	Q	P	L	G	D	K	V	N	F	
GAATGATGGAGTATGGGACCACAAATGGTCAGCTTACCAACCCTTAGGAGATAAGGTCAACT																				
1690																				
F	R	M	V	I	S	N	P	A	A	T	H	Q	D	I	D	F	L	I	E	
TCTTCCGCATGGTCATCTCAAACCCCTGCAGCAACTCACCAAGACATGGACTTCCTCATTG																				
1750																				
E	I	E	R	L	G	Q	D	L	*											
AAGAAATCGAACGCCTGGGACAAGATTGTAATCACTTGTGCTACCAAACTTTCAGTCT																				
1810																				
CTAGGTTAGACAGCTAACGGTGTGCAACAAACTGTGTAAATGTATTGTAGTTGTTCCAGAGT																				
1870																				
AATTCTATTCTATATCGTGGTCAACAGTAGAGTCCAGTTAAA																				
1930																				
1950																				

FIG. 2C

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M A S
 agctcgccgcaagctcgcactcgcaaggcgacctgctccaaaggccatggcatc
 10 S G F W S F G S E D G S G D S E N P
 tccgggtctggcttttgtcttcgggtcggaagatggctctgggattccgagaatcc
 20 G T A R A W C Q V A Q K F T G G I G N K
 cggcacagcgcaaggccctgggtggcaaggtaaaggtaaaggatggctcaccggcgcatggaaacaa
 30 L C A L L Y G D A E K P A E S G G S Q P
 actgtggccctgtctacggagacggccggagaaggccggaaaggccggagggccaaacc
 40 P R A A R K A A C A C D Q K P C S C S
 cccggggccggccggaaaggccggctgcggccctgqgaccagaaggccctgqgagctgctc
 50 K V D V N Y A F L H A T D L L P A C D G
 caaagtggatgtcaactacgcgtttctccatgcaacagacctgctggccggatgg
 60 310 E R P T L A F L Q D V M N I L Q Y V V
 agaaaaggccccactttggcggttgtcaagatggatgttttacatccatgtatggat
 70 320 K S F D R S T K V I D F H Y P N E L L Q
 gaaaaggatcgatagatcaaaccaaaggatgtgattccattatcctaatgaggcttcca
 80 330 E Y N W E L A D Q P Q N L E E I L M H C
 agaatataattggaaattggcagaccaaccacaaaattggaggaaattttgatgcattg
 90 340 Q T T L K Y A I K T G H P R Y F N Q L S
 ccaaacaacttaataatgcaattaaaacagggcatccttagataactcaatcaaacttc
 100 350 T G L D M V G L A A D W L T S T A N T N
 tactggatgtggatgtggatttagcaggactggctgacatcaacagcaaaataactaa
 110 360 550 560 570 580 590

FIG. 3A

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610 N F T Y E I A P V F V L L E Y V T L K K
 catgttccacccatgaaattgttcaggattttgtgccttttggaaatatgtcacactaaagaa
 630
 670 M R E I I G W P G G S G D G I F S P G G
 aatgagagaattcattggctggccaggggctctggcgatggatattttctccgggg
 690
 730 A I S N M Y A M M I A R F K M F P E V K
 cgccatataacatgttatggccatgtatcggcacgcacgcttaaggatgttcccagaatcaa
 770
 790 E K G M A A L P R L I A F T S E H S H F
 ggagaaaaatggctgtttccaggcgttcattggccttcacgtctggaaacataagtcattt
 810
 850 S L K K G A A L G I G T D S V I L I K
 ttctctcaagaaggaggctgcaggcctttagggattttggaaacagacaggcgattgtgatttt
 870
 910 C D E R G K M I P S D L E R R I L E A K
 atgtgatgagaggaaaatgattccatctgatcttggaaaggatttttgaaaggccaa
 930
 970 Q K G F V P F L V S A T A G T T V Y G A
 acagaaagggttttgttcctcgatgtccacaggctggaaaccaccgtgtacggagg
 990
 1030 1050 F D P L L A V A D I C K Y K I W M H V
 atttgacccccctttagctgtgcatttgcacatttggcaaaaaggatataaggatctggatgtcatgt
 1070
 1090 1110 D A A W G G L L M S R K H K W M H V
 ggatgcaggctgggtggggattactgtatgtggaaactgtggaaactgtgg
 1130
 1150 1170 V E R A N S V T W N P H K M M G V P L Q
 cgtggaggccaaactctgtggaaatccacacaagatgtggaggatccctttgca
 1190

FIG. 3B

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C	S	A	L	L	V	R	E	E	G	L	M	Q	N	C	N	Q	M	H	A	1250				
gtgctctgc	t	c	t	c	t	gg	t	ta	g	a	ag	gg	at	tg	c	aa	at	gg	ca	aa	1270			
S	Y	L	F	Q	Q	D	K	H	Y	D	L	S	Y	D	T	G	D	K	A	1310				
ctc	tt	c	tt	c	aa	g	ca	aa	g	at	aa	ac	tt	at	g	ac	act	gg	aa	agg	1330			
L	Q	C	G	R	H	V	D	V	F	K	L	W	L	M	W	R	A	K	G	1350				
ctt	ac	at	gt	gg	ac	cc	ac	gt	tt	tt	aa	ct	at	gg	ct	g	at	tg	gg	gg	aa	agg	1370	
T	T	G	F	E	A	H	V	D	K	C	L	E	L	A	E	Y	L	Y	N	1410				
gact	acc	gg	gg	tt	tt	tg	aa	at	gt	tt	gg	ag	tt	gg	c	ag	gt	tt	tg	at	gg	aa	agg	1430
I	I	K	N	R	E	G	Y	E	M	V	F	D	G	K	P	Q	H	T	N	1450				
cat	ca	ta	aa	aa	ac	cc	ga	aa	gg	at	tg	gg	tt	tg	at	gg	aa	gg	cc	ca	caa	aa	1490	

FIG. 3C

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FIG. 3D

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GAP WEIGHT:	3.000	AVERAGE MATCH:	0.540
LENGTH WEIGHT:	0.100	AVERAGE MISMATCH:	-0.396
QUALITY:	856.2	LENGTH:	585
RATIO:	1.464	GAPS:	0
PERCENT SIMILARITY:	97.436	PERCENT IDENTITY:	96.068

4817, PEP HGT2.PEP

AUGUST 22, 1990 08:20 ..

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1 MASPGSGFWSGSEDGSGDPENPGTARAWCQVAQKFTGGIGNKLCALLY 50
1 MASPGSGFWSGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLY 50
51 DSEKPAESGGSVTSRAATRKVACTCDQKPCSCPCKGDVNAYALLHATDLLPA 100
51 DAEKPAESGGSQPPRCAAACACDQKPCSCSKVDVNAYFLHATDLLPA 100
101 CEGERPTLAFLQDVNMILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150
101 CDGERPTLAFLQDVNMILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150
151 DQPQNLEEILTHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200
151 DQPQNLEEILMHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200
201 NTNMFTEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYA 250
201 NTNMFTEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYA 250
251 MLIARYKMFPEVKKGMAAVPRLIAFTSEHSHFSLKGAAALGIGTDSVI 300
251 MMIAFRKMFPEVKKGMAALPRLIAFTSEHSHFSLKGAAALGIGTDSVI 300
301 LIKCDERGKMIPLSDLERRILEVKQKGFVPFLVSATAGTTVYGAFDPLLAV 350
301 LIKCDERGKMIPLSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAV 350
351 ADICKKYKIWMHVDAAWGGGLLMSRKHKWKLNVERANSVTWNPHKMMGV 400
351 ADICKKYKIWMHVDAAWGGGLLMSRKHKWLNGVERANSVTWNPHKMMGV 400

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FIG.4A

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FIG.4B

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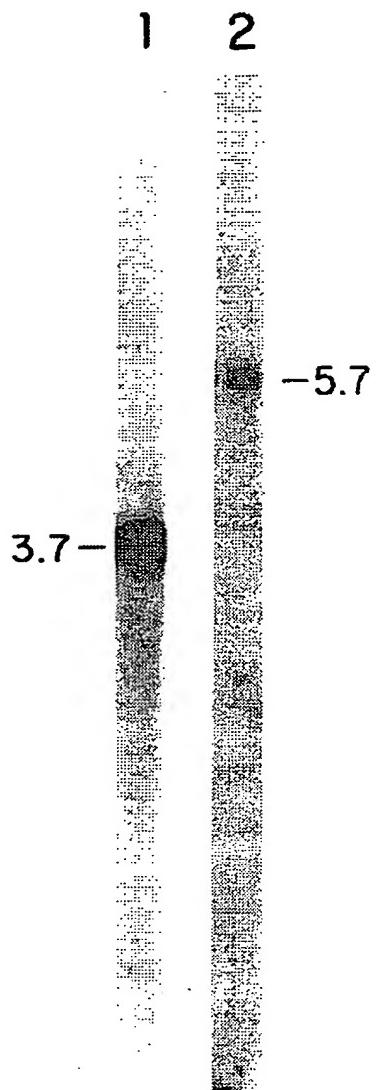


FIG. 5

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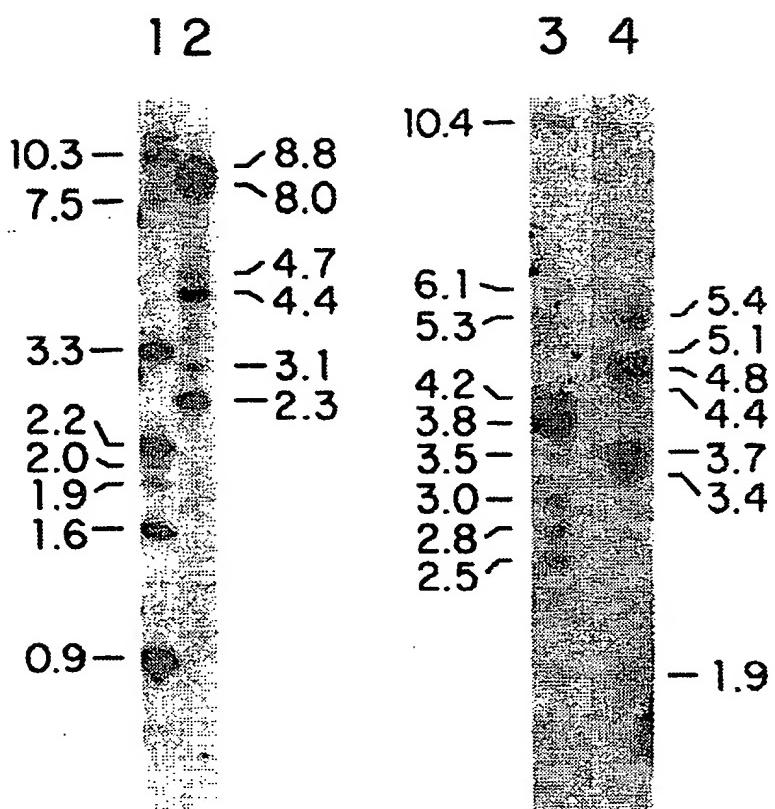


FIG. 6

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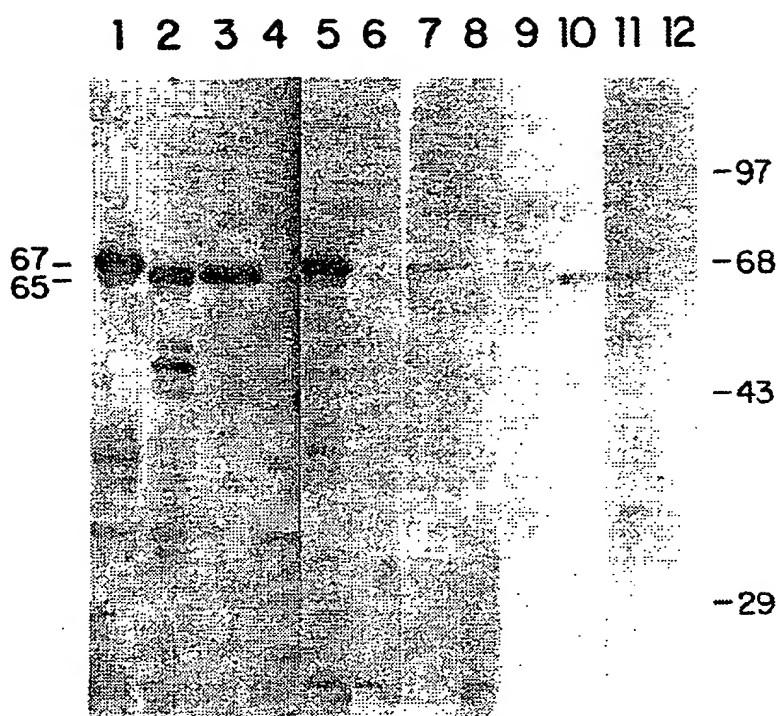


FIG.7

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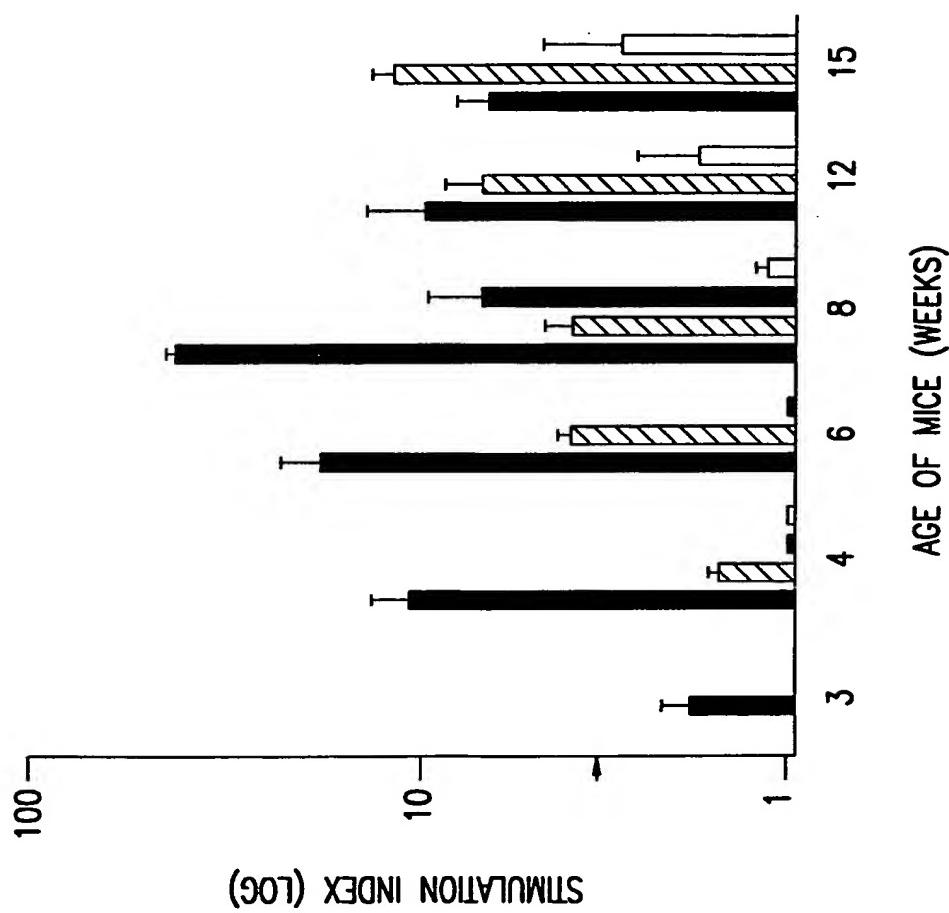


FIG. 8

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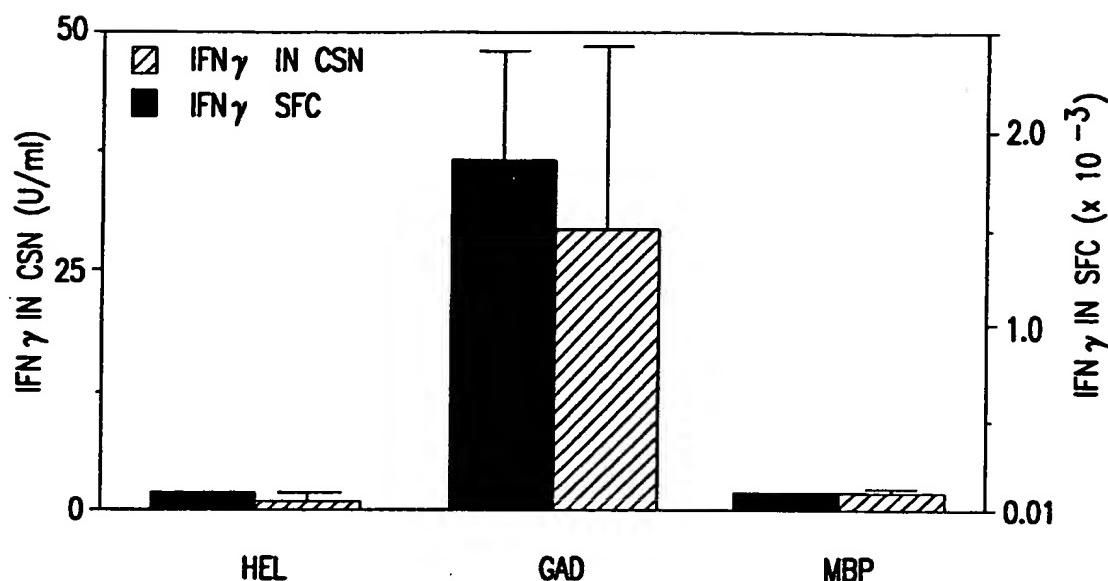


FIG.9A

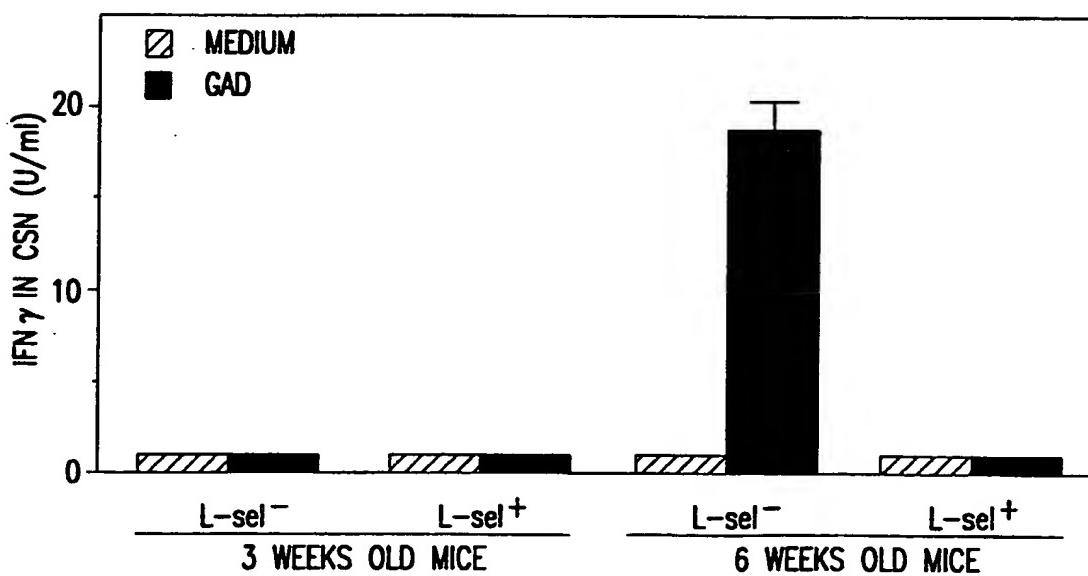


FIG.9B

SUBSTITUTE SHEET (RULE 26)

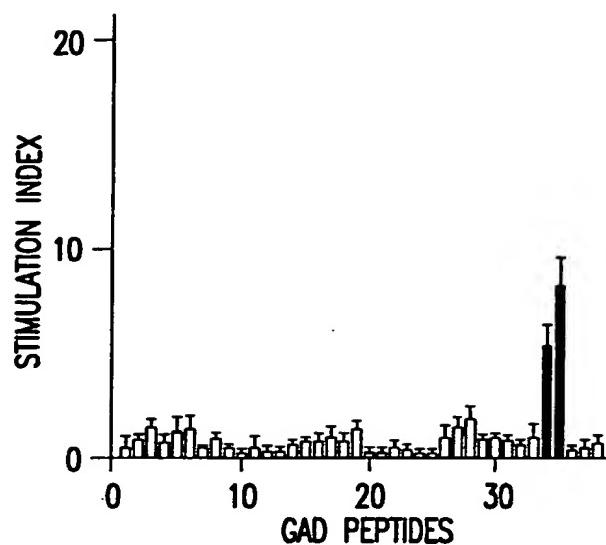


FIG.10A

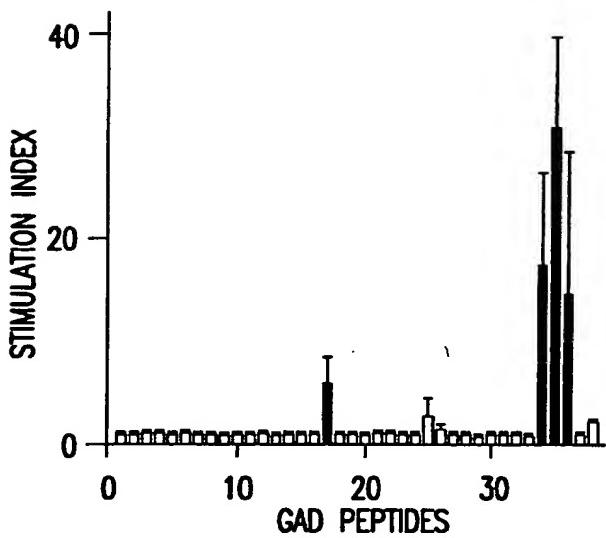


FIG.10B

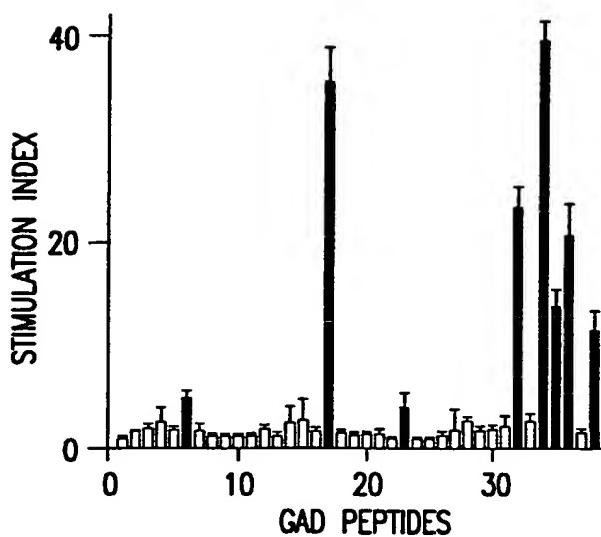
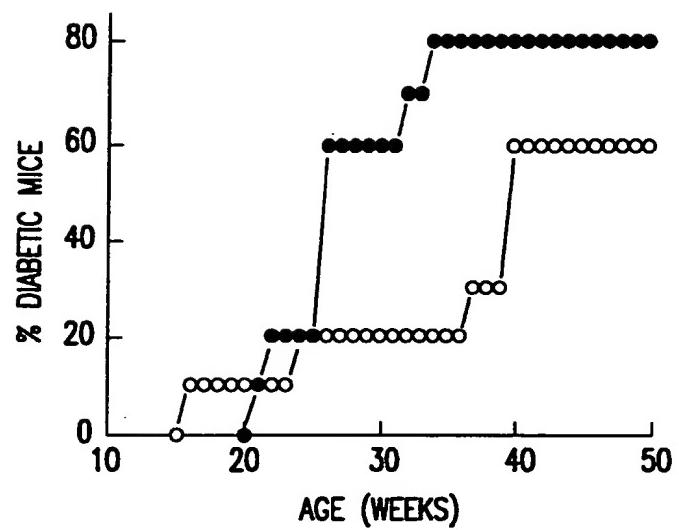


FIG.10C

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- = MICE RECEIVING GAD IN IFA
- = MICE RECEIVING CONTROL β - GAL IN IFA

FIG.11